The effect of nitrogen fertilisation and elevated atmospheric CO_2 concentration on the oxalate content of spinach (*Spinacia oleracea* L.) leaves

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Madhuri Kanala

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Food Science.

The effect of nitrogen fertilisation and elevated atmospheric CO_2 concentration on the oxalate content of spinach (*Spinacia oleracea* L.) leaves

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Oxalate is an anti-nutrient and consuming plants such as spinach that contain high levels of soluble oxalate can have a major effect on calcium bioavailability, leading to the formation of stones in the urinary tract in human beings. This thesis focuses on the effects of nitrogen concentration and form (nitrate, NO_3^- and ammonium, NH_4^+), and elevated atmospheric CO_2 concentration on growth and oxalate levels of spinach leaves grown under greenhouse and controlled environmental conditions. Under greenhouse conditions, the biomass of spinach leaves increased from 1.5 ± 0.3 to 9.2 ± 0.4 g/plant fresh weight (FW) with little change in % dry matter (DM) (27.4 ± 0.2 - 31.4 ± 0.3). The total oxalate levels in spinach leaves decreased from 9.2 ± 0.1 to 3.3 ± 0.1 g/100 g DM with an increase in NO_3^- concentrations from 1-10 mM. Biomass of spinach leaves increased from 1.2 ± 0.2 to 2.9 ± 0.3 g/plant FW with little change in % DM (25.9 ± 0.2 – 27.7 ± 0.2) with an increase in the NH_4NO_3 supply from 1-3 mM. The total oxalate content of spinach leaves decreased from 3.6 ± 1.1 to 2.1 ± 0.3 g/100 g DM with an increase in NH_4NO_3 levels from 1-3 mM.

Under controlled environment conditions at ambient CO_2, biomass of spinach leaves increased from 2.6 ± 0.4 to 13.7 ± 0.7 g/plant FW and % DM increased from 23.9 ± 1.3 - 36.6 ± 1.7 with increased concentration of NO_3^- from 1-10 mM. The total oxalate levels reduced from (10.9 ± 0.1 to 5.1 ± 0.3 g/100 g DM) with increased concentration of NO_3^- supply from 1- 10 mM. The biomass of spinach leaves increased from 2.1 ± 0.3 to 3.8 ± 0.4 with little change in % DM (28.2 ± 0.2 to 32.3 ± 0.2) with increased NH_4NO_3 supply from 1-3 mM. The total oxalate levels reduced from 7.6 ± 0.3 to 5.4 ± 0.4 g/100 g DM with increased concentration of NH_4NO_3 from 1-3 mM. biomass of spinach leaves increased from 7.3 ± 0.3 to 9.7 ± 1.0 g/plant FW with little change in % DM (28.8 ± 0.4 to 30.4 ± 0.6) with increased NH_4NO_3 supply from 1-3 mM. The

total oxalate levels in spinach leaves decreased from 5.0 ± 0.1 to 2.8 ± 0.1 with increased supply of NH_4NO_3 from 1-3 mM.

At elevated atmospheric CO_2, the biomass of spinach leaves increased from 8.1 ± 0.6 to 19.8 ± 1.7 g/plant FW and % DM increased from 29.6 ± 1.7 to 35.7 ± 1.2 with increasing concentrations of NO_3^- from 1-10 mM. The total oxalate levels in spinach leaves reduced from 6.8 ± 0.1 to 2.2 ± 0.3 g/100 g DM with increasing concentrations of NO_3^- from 1-10 mM. The In conclusion, across experiments, growth increased with increased N supply (NO_3^- and NH_4NO_3) and further increased with elevated CO_2 at all N levels. The total and soluble oxalate levels in spinach leaves were negatively correlated with growth with different nitrogen and CO_2 supply across the experiments.

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Table of Contents

List of Tables

List of Figures

Chapter 1
General Introduction

Most green leafy vegetables contain oxalic acid in varying amounts (Morrison & Savage, 2003). Spinach (*Spinacia oleracea* L.) is a widely-consumed green leafy vegetable plant with a high nutrition value but also contains high levels of oxalates, an anti-nutritive factor. Spinach is a rich source of calcium, iron, vitamins and other essential nutrients. However, spinach contains high levels of oxalates, an anti-nutritive component (Savage *et al.,* 2000; Morrison & Savage, 2003; Siener *et al.,* 2006; Savage & Mårtensson, 2010). Oxalic acid occurs as a product of metabolism in plant tissues and is found in the form of soluble or insoluble oxalate. Oxalic acid and its salts, oxalates, are produced as end products of metabolism in a variety of plants. Oxalic acid forms water-soluble salts with sodium, potassium and ammonium ions, and insoluble salts with calcium, iron and magnesium ions and, thereby, decrease the bioavailability of many such minerals that are essential for the human body (Noonan & Savage, 1999).

Carbon dioxide and light are required for photosynthesis and hence determine plant production and also play a role in determining plant quality. Plants in their juvenile phase can grow exponentially compared to mature plants (Tremblay & Gosselin, 1998). Along with CO_2, plants need additional nutrients in the form of fertilisers. Nitrogen is an essential nutrient for plants and is required in the largest quantity from the soil (Hafia Group, 2019; Crop Nutrition 2019). In cultivated soils, plants take up most of the nitrogen in the form of NO_3^- or NH_4^+ (Summit Fertilisers, 2019). Application of nitrogen (NO_3^- and NH_4^+) increases proteins as nitrogen is a major component of amino acids and also increases other nutritional elements such as iron, potassium and calcium (Andrews *et al.,* 2013).

Increased levels of CO_2 in the atmosphere can increase plant growth by providing a 'fertilisation' effect (Hamada *et al*., 2016). Giri *et al.* (2016) stated that rising atmospheric CO_2 levels could have a negative impact on the nutritional quality of spinach. Elevated CO_2 depresses the concentration of several nutrients which are essential for human nutrition in the edible parts of these plants. It decreases the level of nitrogen, phosphorus and potassium along with many crucial micronutrients including copper, zinc, magnesium and sulphur in spinach. The significant decline in nutritional quality in leafy vegetables when treated with elevated CO_2 may signify a potential for a wide-spread dietary nutrient deficiency, especially as fruits and vegetables are becoming a significant part of our daily diet. Rising CO_2 levels can compromise the overall nutritional quality by depressing the concentration of many essential

1.1 Objectives of this study

The objectives of this study were to determine-

1. The effects of nitrogen concentration and form (NO_3^- and NH_4^+) on growth and oxalate levels in spinach leaves.
2. The effect of elevated atmospheric CO_2 on growth and oxalate levels in spinach leaves.
3. If there is an interaction between nitrogen form and concentration and atmospheric CO_2 concentration on growth and oxalate levels in spinach leaves.

   The experiments were carried out under greenhouse and controlled environmental conditions.

Chapter 2
Review of the literature

2.1 Nutritional and anti-nutritional profile of spinach

Worldwide Spinach (*Spinacia oleracea* L.) is an economically important vegetable crop and is considered one of the healthiest vegetables in the human diet. Spinach has a high concentration of nutrients and health-promoting compounds such as beta carotene (provitamin A), lutein, folate, vitamin C, calcium, iron, phosphorous, and potassium (Dicoteau, 2000; Morelock & Correll 2008; Correll *et al.* 2011; Lester *et al.* 2013). Spinach is a widely consumed leafy vegetable with a high nutritional value but also contains high levels of oxalates, an anti-nutritive factor (Noonan and Savage, 1999). Oxalate in spinach is known to have a major effect on calcium bioavailability. However, the concentration of oxalates varies widely in plants based on the cultivar, treatment type and environmental factors (Noonan & Savage, 1999). While some cultivars of spinach contain oxalate in the range of 700-900 mg/100 g on a fresh weight basis (Morrison & Savage, 2003; Brogren & Savage, 2003), other cultivars have about 1700 mg/100 g on a fresh weight basis (Savage *et al.,* 2000).

2.2 Bioavailability and classification of oxalates

Oxalates provide defence and protection to plants. While oxalic acid is a natural product of mammalian metabolism, the consumption of additional oxalic acid may cause stone formation in the urinary tract in humans (Noonan & Savage, 1999). Foods that contain high levels of oxalate include spinach, beets, wheat bran, rhubarb, black tea and nuts (Charrier *et al.,* 2002; Brogren & Savage, 2003; Savage *et al.,* 2003). However, the oxalic acid contents vary with plant cultivars, environmental factors and the fraction of the plant (Noonan & Savage, 1999). The potential adverse effects of oxalate on the bioavailability of calcium in humans is determined by the molar ratio of oxalate to calcium. Based on oxalate to calcium ratio, Noonan & Savage (1999) categorised foods that contain oxalate into three major groups: 'Group 1' includes foods that have oxalate to calcium ratio of higher than two, 'Group 2' foods with ratio ranging between 1.0 and 2.0 and 'Group 3' foods that have an oxalate to calcium ratio of lesser than one.

When consuming plants that contain high levels of dietary soluble oxalate, the oxalate binds to calcium from other foods consumed at the same time. This allows the formation of insoluble oxalate

thereby reducing the absorption of calcium and other minerals from the intestine (Ghosh Das & Savage, 2013). However, the small intestine absorbs higher amounts of soluble
oxalates which increases the chances of stone formation in the kidney (Noonan & Savage, 1999).

2.3 Biosynthesis and toxicity of oxalates in humans

Most studies suggest that the major site for oxalate absorption in humans is in the small intestine (Holmes *et al.,* 1995; Simpson *et al.,* 1999; Savage & Mårtensson, 2010). The rate at which oxalate is absorbed and the amount of oxalate absorption vary based on individual diets (Noonan & Savage, 1999). However, the absorption of oxalate from dietary sources is relatively low. In most individuals, the levels of absorbed oxalates are only a fraction of the total oxalate ingested and are estimated to be in the range of 5-15% depending on the co-ingestion of dietary fibre, calcium and magnesium in the foods (Noonan & Savage, 1999; Savage & Mårtensson, 2010). Oxalate is broken down by enzymes in the colon or oxalate degrading bacteria in the gastrointestinal tract (Savage & Mårtensson, 2010). Anaerobic *Oxalobacter formigenes* is the oxalate degrading bacterium that influence the absorption of oxalate in the gastrointestinal tract (Kwak *et al.,* 2003). This bacterium uses oxalate as an energy-yielding substance for growth. On an average, individuals prone to form kidney stone absorb up to 50% more oxalate than normal individuals (Hesse *et al.,* 1999; Chai & Liebman, 2005; Voss *et al.,* 2006).

The two major effects of consuming high oxalate containing foods are; firstly, oxalic acid can form insoluble salts by binding to minerals such as calcium, magnesium, iron etc. in the foods and, thereby, decrease the bioavailability of many such minerals that are essential for the human body (Noonan & Savage, 1999); the insoluble oxalate salts that are not absorbed are excreted in the faeces (Marengo & Romani, 2008). Secondly, soluble oxalate, once absorbed, cannot be used in the body and needs to be excreted. In the process of removing soluble oxalate from venous blood, filtered by the kidneys, soluble oxalate may become insoluble oxalate by being bound to calcium. This accumulation of calcium oxalate in the kidneys can lead to the formation of kidney stones (Williams & Wandzilak, 1989; Noonan & Savage, 1999; Chai & Liebman, 2005). Majority of kidney stones formed are calcium oxalate stones. This could be due to inherited factors, diet and/or environmental factors. The diet is primarily considered an important environmental factor that largely influences kidney stone formation (Grifinn, 2004; Worcester & Coe, 2008; Vani *et al.,* 2009). Avoiding large quantities of high oxalate foods and consuming calcium-rich foods such as dairy products along with oxalate containing foods can prevent the formation of calcium oxalate stone (Mårtensson & Savage, 2008).

2.4 Effect of nitrogen fertilisation on spinach yield and nutrition

Spinach responds greatly to nitrogen fertilisation (Magnifico *et al.,* 1992; Cantliffe, 1992). Due to its efficient uptake system, spinach plants accumulate high amounts of NO_3^- (Maynard *et al.,* 1976). Several studies on the effect of nitrogen fertilisation on growth and oxalate accumulation have shown that most leafy vegetables responded better to NO_3^- when compared to NH_4NO_3, and have higher growth rates (Zhang *et al.,* 1990, Van der Boon *et al.,* 1990, Santamaria & Elia, 1997; Wang & Li, 2003).

2.5 Effect of elevated atmospheric CO_2 on plant yield and growth

According to the National Oceanic and Atmospheric Administration (NOAA), data recorded in March 2018 at Mauna Loa observatory, the level of atmospheric CO_2 was at 405.56 ppm (atmospheric CO_2 level recorded in July 2019 was 411.77 ppm). Plants need CO_2 to survive. Plants in their juvenile phase can grow exponentially, compared to mature plants. CO_2 and light are needed to carry out photosynthesis and carbon fixation (Tremblay & Gosselin, 1998). Various studies suggest that increased levels of atmospheric CO_2 concentrations lead to lower transpiration rates and thicker leaves. Genrally the levels of atmospheric CO_2 concentration and light in controlled growth conditions is beneficial for growing crops (Tremblay & Gosselin, 1998). Increased levels of CO_2 in the atmosphere can elevate plant growth by increasing photosynthesis (Hamada *et al*., 2016).

With twice the concentration of CO_2 in the atmosphere, crop yields increase by 33% on an average. Generally, CO_2 levels in greenhouses vary between 700-900 ppm (Kimball, 1983). Vegetable plants enriched with CO_2 reduce the time of growth and alter photosynthate allocation to various parts of the plant. This leads to plants which are sturdier (Kimball, 1983). CO_2 enrichment of plants also increases water use efficiency and this subsequently improves growth (Andrews *et al.,* 2019).

However, Thayer (2016) stated that at a particular point excess CO_2 enrichment can have adverse effects on plant growth rate. The study showed the relationship between the level of CO_2 enrichment and plant growth rate. With levels lower than 200 ppm, plants did not have sufficient CO_2 to go through the process of photosynthesis and will effectively stop growing.

The study also demonstrates that when the levels of atmospheric CO_2concentration is doubled, it also doubles the growth rate.

2.6 Effect of elevated atmospheric CO_2 on plant nutrition

With recent advances in technology, several experiments have been conducted using Free Air Concentration Enrichment (FACE) which have provided valuable understanding of the increasing levels of CO_2 concentration in the atmosphere and its relationship with plant growth Myers *et al.* (2014). Increase in the concentration of CO_2 level in the atmosphere aids in stimulating nitrogen fixation, especially amongst plants that form a symbiotic relationship with nitrogen-fixing bacteria. As the ambient CO_2 rises, it is more likely to increase the availability of nitrogen in the soil, subsequently leading to larger plant productivity. With an increase in the atmospheric CO_2 levels, most plants respond with high rates of biomass production and a higher rate of photosynthesis. Increase in the number of finer roots and the overall root surface area helps in increasing the total uptake of nutrients under enriched CO_2 conditions. This stimulates the development and growth of plants (Ferguson, n.d).

Myers *et al.* (2014) conducted research on various cultivars of rice, wheat, maize and soybean using FACE. Their study reveals that under enhanced CO_2 conditions there was a decrease in the nutritional quality of the plants. The results showed more than tenfold decrease in iron and zinc in the edible parts of the plants. Legumes grown explicitly under field conditions with enhanced CO_2 had lower concentrations of iron and lower amounts of protein. Although increased levels of CO_2 make carbon more available, plants require various other nutrients such as phosphorus and nitrogen to survive. Imbalance in the levels of these nutrients can eventually decrease the nutritional quality of the plant.

Experiments conducted on wheat, rice and potato show a 5-14% decrease in the levels of protein when grown under enriched CO_2 conditions (Taub, 2008). Feng *et al.,* (2015) at the University of Gothenberg, Sweden, stated that increased concentrations of CO_2 affect the ability of plants to actively absorb nutrients, regardless of how effective the plant growth rate is. Enhanced CO_2 reduces nitrogen absorption and results in a dilutive effect with regard to plant nutrients. The primary reason is that the nitrogen absorption rate is much slower when compared to the plant growth and photosynthesis rate, under enriched CO_2 conditions.

2.7 Effect of elevated atmospheric CO_2 on spinach growth and oxalate levels

Rising atmospheric CO_2 levels can have a negative impact on the nutritional quality of spinach. It decreases the concentration of nitrogen, phosphorus and potassium along with many essential micronutrients including copper, zinc, magnesium and sulphur in spinach. Rising CO_2 levels can compromise the general nutritional quality by depressing the concentration of many crucial nutrients in commonly consumed leafy vegetables such as spinach (Giri *et al.,* 2016).

Proietti *et al.,* (2013) evaluated the growth conditions and the resulting oxalate content of spinach grown in a plant chamber and fertilised with CO_2. Plants grown with high CO_2concentrations had greater weight on a fresh matter and dry matter basis. CO_2 enriched plants also had a large leaf area. Although elevated CO_2 increases plant growth rate substantially, it does not necessarily have a positive effect on the nutrition in the plants (Myers *et al.*, 2014; Li *et al.,* 2018). Increased CO_2 levels could lower the levels of calcium, iron, zinc and phosphorous (Dietterich *et al.,* 2015). Likely, this could also lead to lower levels of antinutrients concentration, such as oxalates. Proietti *et al.* (2013) reported that increasing CO_2 concentration led to an increase in growth and a decrease in oxalic acid levels. This report needs to be verified. Also, the interaction between nitrogen forms and concentrations and elevated atmospheric CO_2 concentration on total and soluble oxalate levels in spinach leaves has been little studied.

2.8 Conclusion

Spinach is a leafy vegetable that is easy to grow in many different environmental conditions. It is also widely consumed in many cultures because it contains rich source of calcium, iron vitamins and many other nutrients. Unfortunately, the leaves also contain significant levels of oxalates which can bind to calcium, making this important mineral unavailable. The effect of consuming soluble oxalates in the diet is also widely understood.

Plants use CO_2 as their source of carbon and increasing the concentration of CO_2 is widely used in greenhouses to increase the growth rate of many different food plants. However, increasing atmospheric CO_2 levels can have a negative impact on the nutritional quality of spinach; the concentration of nitrogen, phosphorus and potassium along with many important micronutrients including copper, zinc, magnesium and sulphur appear to be reduced in spinach leaves under these conditions.

The effect of nitrogen and increased levels of CO_2 in the atmosphere on plant growth have been studied but the effect on oxalate levels has not extensively been investigated. Therefore, research was undertaken to examine the effect of increasing levels of NO_3^- or NH_4NO_3 on growth and oxalate levels of spinach leaves grown under greenhouse and elevated atmospheric CO_2 condition.

Chapter 3

Growth and oxalate levels of spinach fertilised with NO_3^- or NH_4NO_3 and grown under greenhouse conditions

3.1 Introduction

Spinach (*Spinacia oleracea* L.) is a nutrient-rich leafy vegetable consisting of large amounts of several essential minerals, vitamins, flavonoids and folic acid (Kawazu *et al.,* 2003; Kaminishi & Kita, 2006). Spinach also contains high levels of oxalate, which is an anti-nutrient (Siener *et al.,* 2006)**.** Oxalate is distributed widely in the plant kingdom, and several species of plants have large concentrations in their leaves. Spinach, in particular, contains 5-15% total oxalate in leaves on a DM basis (Cai *et al.,* 2018). Oxalate is known to play important functional roles in plants. Plants produce oxalates as an end metabolic product. Oxalates occur in two forms in plants; soluble oxalates which are bound to Na^+, K^+ and NH_4^+ and insoluble oxalates which are commonly bound to Ca^{2+}, Mg^2+ and Fe^{2+} (Savage *et al.,* 2000). Soluble oxalates from foods that remain unbound are readily absorbed in the gastrointestinal tract but, as oxalates cannot be utilised in the body, they are quickly excreted by the kidneys. When oxalates are excreted, they can combine with calcium that is being excreted at the same time and this leads to the formation of kidney stones.

Oxalate accumulation in plants depends on various factors, such as cultivar traits, nutrient management and species (Libert & Franceschi, 1987; Rassam & Laing, 2005). Oxalate content differs among genotypes, even within a given species for many vegetable crops (Siener *et al.,* 2006; Szalai *et al.,* 2010). This is also true for spinach (Mou, 2008; Koh *et al.,* 2012). According to Brogren & Savage (2003), frozen commercially available spinach contains 956.7 ± 83.5 mg/100 g total oxalate and 736.6 ± 20.4 mg/100 g soluble oxalate on a FW basis. According to another study conducted by Radek and Savage (2008), on a DM basis, total oxalate in spinach leaves was 12,576.2 ± 107.9 mg/100 g and soluble oxalate was 11,899.8 ± 181.2 mg/100 g. Oxalate content in spinach varies with genotype. Kaminishi and Kita (2006) stated that spinach cultivars with higher growth rate contain higher NO_3^- levels and lower levels of oxalate and cultivars with lower growth rates contain lower NO_3^- levels and higher oxalate levels. According to Mou (2008), the total oxalate of spinach ranged between 53.4-116.2 mg/100 g DM when grown in a greenhouse using standard potting mixture and fertilised with NO_3^- at Salinas, California.

Nutrient nitrogen is one of the most important agronomic factors affecting the oxalate content in plants. Previous studies have indicated that the application of nitrogen of different forms, such as NO_3^- or NH_4^+, could affect the levels of oxalate in plants (Al Daini *et al.;* 2013; Liu *et al.,* 2014; Lin *et al.*, 2014). Plants that had been treated with NO_3^- were found to have accumulated higher oxalate levels than those that had been fertilised with NH_4^+. NH_4^+ fertiliser significantly reduced the oxalate accumulation in plants by inhibiting the uptake of NO_3^- (Al Daini *et al.;* 2013; Liu *et al.,* 2014; Lin *et al*., 2014). The NO_3^- inhibited the enzymatic activity of oxalic acid oxidase and resulted in the accumulation of oxalic acid in the leaves and stems (Libert & Franceschi, 1987; Palaniswami *et al.,* 2004).

Plants usually take up nitrogen in the form of NO_3^- and NH_4^+, therefore, the total N absorbed consists of a combination of these two forms (Briones *et al.,* 2003; Vasileva & Ilieva, 2011). The ratio of NO_3^- to NH_4^+ is of great significance and can have an impact on plant growth. The optimum growth of plant species usually requires a mix of NO_3^- and NH_4^+. A suggested strategy to reduce oxalate accumulation in plants is the management of fertilisers (Ji & Peng, 2005; Lin *et al.,* 2014). Nitrogen concentration and form is one of the most important agronomic factors affecting oxalate contents in plants (Palaniswamy *et al.,* 2004). Some reports have suggested that application of nitrogen of different forms, NO_3^- and NH_4^+, appeared to be one way to effectively regulate oxalate levels in plants (Ji & Peng, 2005; Al Daini *et al.,* 2013).

The present study focuses on the effects of nitrogen concentrations and form (NO_3^- and NH_4^+) on growth and oxalate levels of spinach leaves grown under greenhouse conditions.

3.2 Materials and methods

Spinach plants *(S. oleracea.* L) were grown in one litre plant pots containing a standard N-free growing mixture (media: 1.3 L bark and 0.3 L pumice (Intelligro, Christchurch, New Zealand); fertilisers: 0-0-37 0.5 g Scotts Osmocote (Evergreen Garden Care, New South Wales, Australia), 1.7 g horticultural lime (Southern Horticultural Products Ltd, Christchurch, New Zealand), 0.5 g superphosphate, 0.5 g micromax and 1.7 g hydraflo (ICL Speciality Fertilizers, Tel Aviv, Israel) for five weeks in a greenhouse with temperature ranging from 15- 25°C at the Horticulture Research Area, Lincoln University, Canterbury, New Zealand (43° 38' 43" S, 172° 27' 43" E), 10 m above sea level. The seeds were sourced from Kings Seeds, Katikati, Bay of Plenty, New Zealand. Five spinach seeds were sown into each pot on the 25th of December

2016, and once the spinach seeds had sprouted, the plants were reduced to two plants per pot on the 7th of January 2017. One group of spinach plants were fertilised with different concentrations of NO_3^- and another group of spinach plants were fertilised with different concentrations of NH_4NO_3 (Table 3.1). There were five replicate pots for each concentration of NO_3^- and NH_4NO_3. Each concentration of NO_3^- and NH_4NO_3 were made up to 2000 mL volume with tap water. Each pot was flushed through with 200 mL of the above solution twice a day (morning and evening).

Table 3.1 Nitrogen concentration and form, amount used (g/100 g), number of replicates and rate of dilution used in growing spinach plants under greenhouse conditions.

Nitrogen form	Concentrations (mM)	Amount added to each plant pot twice a day (g/100 g)	Replicates	Dilution
NO_3^-	1	0.20	Five pots per treatment	Each concentration made up to 2000 mL with tap water
	2	0.40		
	3	0.60		
	4	0.80		
	6	1.20		
	8	1.60		
	10	2.00		
NH_3NH_4	1	0.12	Five pots per treatment	Each concentration made up to 2000 mL with tap water
	2	0.24		
	3	0.32		

3.2.1 Harvesting

Spinach plants were harvested at the end of week five (29th January 2017). The whole plant was washed with cold tap water to remove soil particles and excess water was dabbed with a paper cloth.

3.2.2 Biomass and sample preparation

The whole plant weight of each spinach plant was recorded. The leaves, roots and stems of each spinach plant were weighed separately. Spinach leaves produced from each plant was used to determine the biomass. The leaves were then divided into three paper bags per NO_3^- or

NH_4NO_3 concentration and dried. The dried leaves were ground into a fine powder using a mortar and pestle, and approximately 0.4 g of the powdered leaf sample was used for analysis.

3.2.3 Dry matter

The dry matter (DM) contents of spinach leaves were determined by drying in an oven (Watvic, Watson Victor Ltd., New Zealand) to a constant weight at 105°C for 24 hours, following a method outlined by Ruiz (2001).

3.2.4 Extraction of total and soluble oxalic acid

The measurement of total and soluble oxalates was performed following the method outlined by Savage *et al*. (2000). Three replicates of each sample (0.4 g per sample x three replicates) were extracted to measure total and soluble oxalate contents. 40 mL of 0.2 M HCL (Aristar, BDH Chemicals, Ltd., Poole, Dorset, UK) was added to volumetric flasks for the total oxalate extraction and also 40 mL of Nanopore II water (Barnstead International, Dubuque, Iowa, USA, 18 MΩ cm) was added for the extraction of soluble oxalates (Savage *et al.,* 2000). All flasks were placed in an 80°C shaking water bath for 20 minutes. The solutions were allowed to cool to 20°C and then made up to 100 mL with 0.2 M HCL for total oxalate and Nanopore II water for soluble oxalate, respectively.

3.2.5 Sample analysis

The extracts in the volumetric flasks were filtered through a cellulose acetate syringe filter with a pore size of 0.45 µm (dismic-25cs, Advantec, California, USA) into 1 mL glass HPLC vials. The samples were analysed with a high-performance liquid chromatography (HPLC) system, using a 300 mm x 7.8 mm Rezex ion exclusion column (Phenomenex Inc., Torrance, CA, USA) attached to a Cation-H guard column (Bio-Rad, Richmond, CA, USA) held at 25ºC. The analysis was performed by injecting 20 µL of sample or standard onto the column using an aqueous solution of 25 mm sulphuric acid (HPLC grade Baker Chemicals, Phillipsburg NJ, USA) as the mobile phase, then pumped isocratically at 0.6 mL/min, with peaks detected at 210 nm. The HPLC equipment consisted of a Shimadzu LC-10AD pump, CTO-10A column oven, SPD-10Avp UV-Vis detector (Shimadzu, Kyoto, Japan) and a Waters 717 plus autosampler (Waters, Milford MA, USA). Data acquisition and processing were undertaken using the Peak Simple Chromatography Data System (model 203) and Peak Simple software version 4.37 (SRI Instruments, Torrance CA, USA). The oxalic acid peak was identified by comparing the retention time to a standard solution and by spiking an already-filtered sample containing a known amount of oxalic acid standard. The insoluble oxalate content of each sample was calculated by the difference between the total and the soluble oxalate contents (Holloway *et al.,* 1989).

3.2.6 Standard calibration

Two standard curves of oxalic acid (99.99% oxalic acid, Sigma-Aldrich Co., St. Louis, USA) were analysed, using standards of the following concentrations: 1, 2, 5, 10, 15 and 25 mg/100 mL. One batch of standards were prepared in 0.2 M HCL while the other was prepared in Nanopore II water. The acid standard curve was used for identifying and calculating the total oxalate content, while the water standard curve was used for the soluble oxalate content (Appendix 1). All blank and standard solutions were passed through a 0.45 µm cellulose acetate filter before analysis.

3.2.7 Statistical analysis

All calculations were performed using Excel 2016, and statistical analysis was carried out using Minitab Statistical Software (USA) (version 18.1) for Windows 10 (USA). A General Linear Model (GLM) was used to perform analysis of variance (ANOVA). All effects described as significant have a probability (P) value < 0.001 or $P < 0.05$. A linear regression analysis was carried out on data obtained from experiments. The data from the results are presented as mean ± standard error (SE).

3.3 Results and Discussion

3.3.1 Biomass and oxalate levels of spinach leaves grown with different NO_3^- concentrations under greenhouse conditions

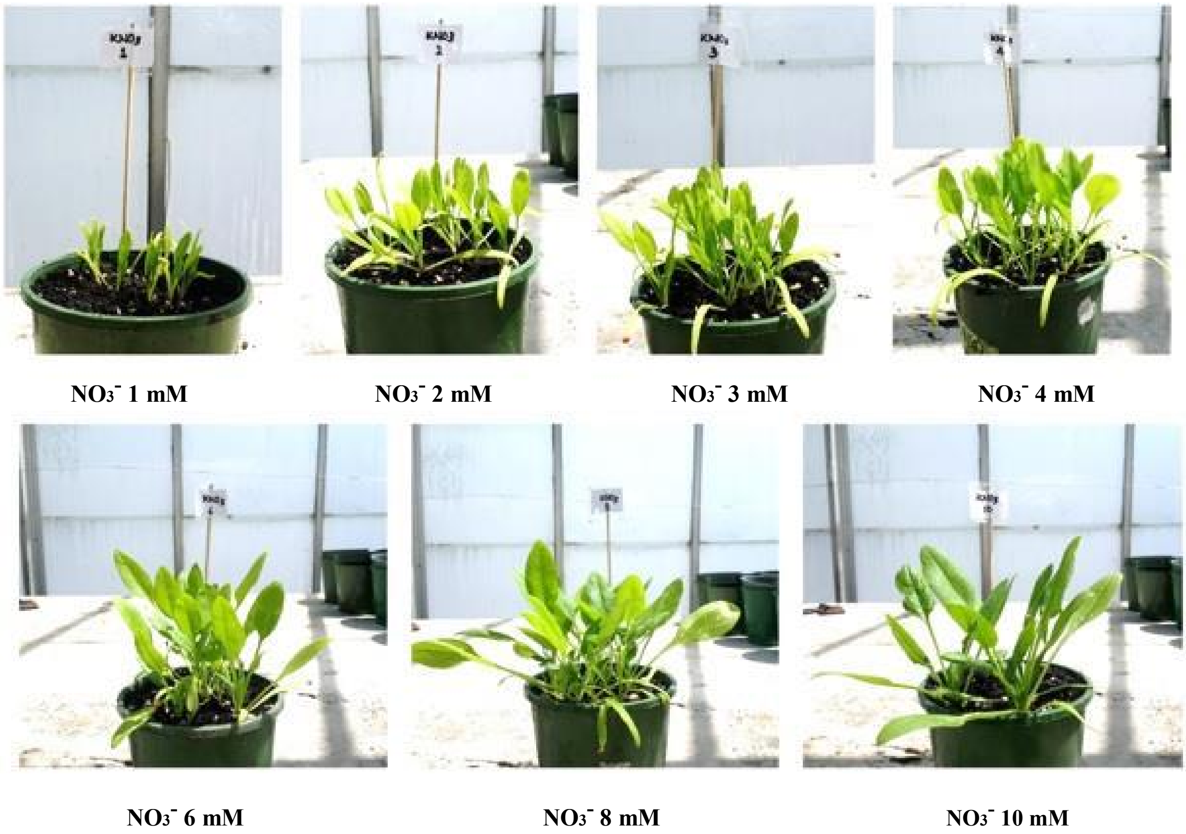

Figure 3.1 Spinach plants after two weeks of growth under different NO_3^- supply in a greenhouse

Nitrogen is the most abundant nutrient element taken up from soil and subsequently removed in vegetable crop harvests (Bishop *et al*., 1972; Cutcliffe & Munro 1976; Brandenburg 1980). In this study there was a substantial increase in the growth of spinach plants with increased NO_3^- concentrations from 1-10 mM and this is clearly shown in the photograph in Fig 3.1. Specifically there was a linear increase in biomass of spinach leaves with an increase in NO_3^- concentration from 1-10 mM (Fig 3.2 and Table 3.2).

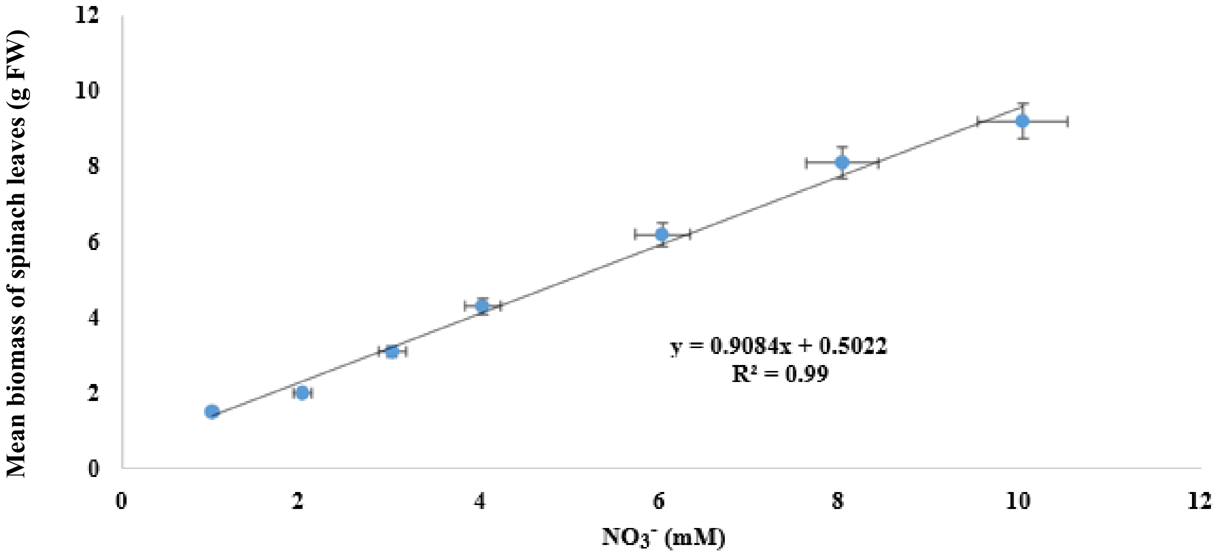

Figure 3.2 Biomass of spinach leaves (mean of ten plants FW) grown with different concentrations of NO_3^- under greenhouse conditions.

The biomass of spinach leaves increased linearly from 1.5 ± 0.3 to 9.2 ± 0.4 g/plant FW with little change in % DM (27.4 ± 0.2 - 31.4 ± 0.3) with an increase in NO_3^- concentration from 110 mM (Fig 3.2 and Table 3.2). Therefore, the dry weight (DW) of spinach leaves also increased with increased NO_3^- supply. There are many reports of increased growth of crop plants including spinach over this range of NO_3^- concentration (Andrews *et al.,* 2013). Generally, growth of crop plants reaches a maximum over this NO_3^- range but this did not occur with spinach.

Table 3.2 Biomass and mean total, soluble and insoluble oxalate (g/100 g ± SE DM) (mean values of ten plants) grown with different NO_3^- concentrations under greenhouse conditions. Values in brackets are % soluble oxalate of total oxalate.

Plants watered with NO_3^- concentrations (mM)	Biomass of spinach leaves produced after five weeks of growth (g/plant FW)	Oxalate (g/100 g DM)		
		Total	Soluble	Insoluble
1	1.5 ± 0.3	9.2 ± 0.4	4.7 ± 0.1 (51)	4.5 ± 0.3
2	2.0 ± 0.2	8.4 ± 0.5	4.1 ± 0.1 (47)	4.3 ± 0.4
3	3.1 ± 0.3	6.4 ± 0.1	3.0 ± 0.1 (47)	3.4 ± 0.0
4	4.3 ± 0.4	5.2 ± 0.1	3.2 ± 0.1 (62)	2.0 ± 0.0
6	6.2 ± 0.5	5.1 ± 0.1	3.0 ± 0.1 (59)	2.1 ± 0.0
8	8.1 ± 0.5	4.5 ± 0.2	1.5 ± 0.1 (33)	3.0 ± 0.1
10	9.2 ± 0.4	3.3 ± 0.1	2.3 ± 0.0 (70)	1.0 ± 0.1

Analysis of Variance	df	Biomass	Total	Soluble	Insoluble
Fertiliser treatment	**6**	***	***	***	***

Significance: ***P < 0.001

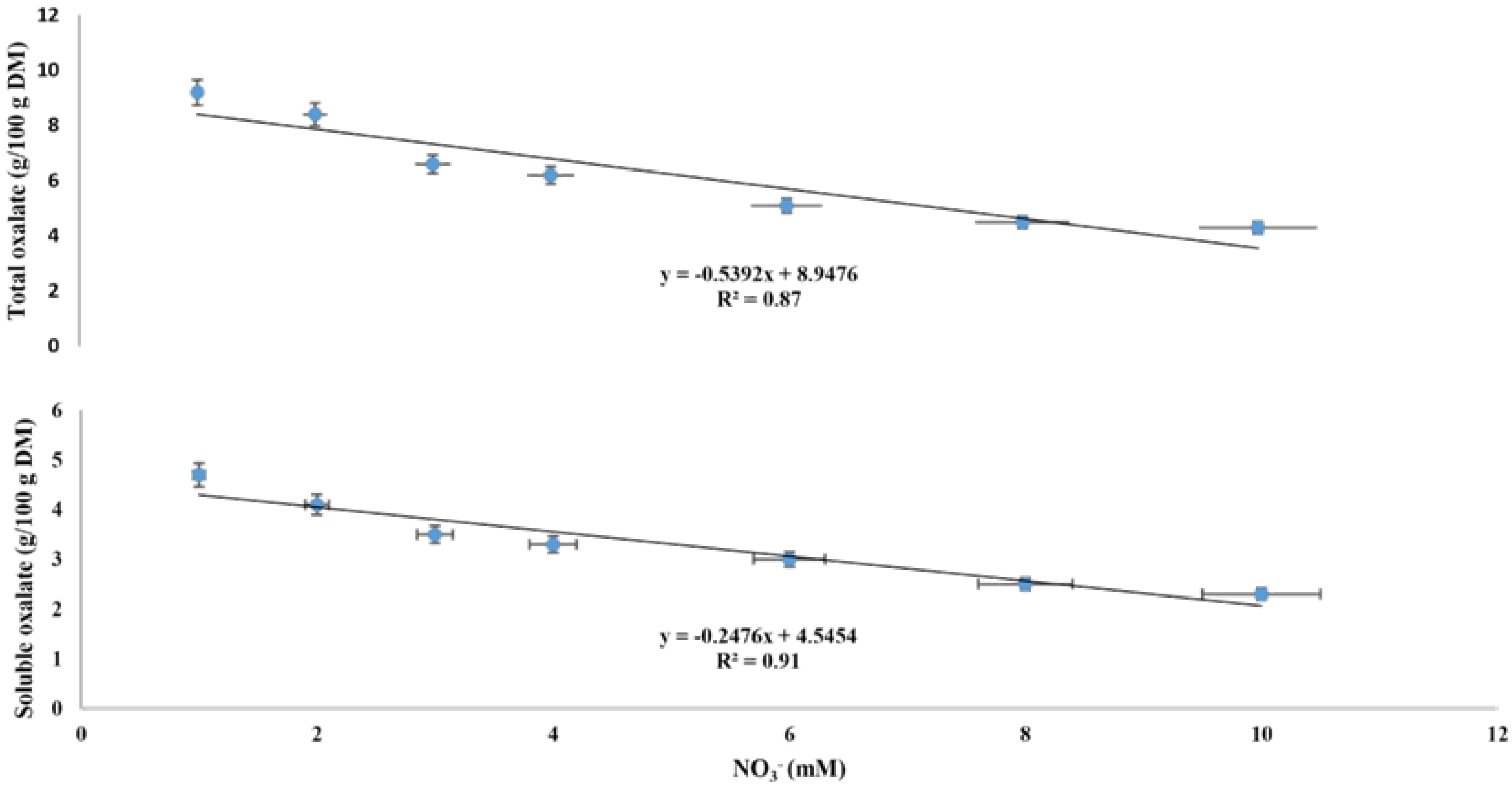

Figure 3.3 Total and soluble oxalate of spinach leaves (g/100 g DM) fertilised with different concentrations of NO_3^- and grown under greenhouse conditions.

The total oxalate (9.2 ± 0.1 to 3.3 ± 0.1 g/100 g DM) and soluble oxalate (4.7 ± 0.2 to 2.3 ± 0.1 g/100 g DM) levels in spinach leaves decreased linearly with an increase in NO_3^- concentration from 1-10 mM (Table 3.2 and Fig 3.3). Total oxalate and soluble oxalate reduced by 64% and 51% respectively with an increase in NO_3^- concentration from 1-10 mM. Increasing NO_3^- concentration from 1-10 mM resulted in a negative correlation between the levels of total and soluble oxalate in spinach leaves and leaf growth. The percent soluble oxalate of total oxalate in spinach leaves ranged between 33 and 70.

3.3.2 Biomass and oxalate levels of spinach leaves grown with different NH_4NO_3 concentrations and grown under greenhouse conditions.

NH_4NO_3 1 mM NH_4NO_3 2 mM NH_4NO_3 3 mM

Figure 3.4 Spinach plants after two weeks of growth under different NH_4NO_3 supply grown in a greenhouse.

Some reports have suggested that application of nitrogen in different forms, e.g., NO_3^- and NH_4^+ can be used to effectively regulate oxalate levels in plants (Ji & Peng, 2005; Al Daini *et al.,* 2013). There was a substantial increase in the growth of spinach plants with an increase in NH_4NO_3 concentration from 1-3 mM and this is clearly shown in the photograph in Fig 3.4. Specifically, biomass of spinach was twice as great at 3mM NH_4NO_3 as at 1 and 2 mM NH_4NO_3 (Fig 3.5 and Table 3.3). Biomass of spinach leaves increased from 1.2 ± 0.2 to 2.9 ± 0.3 g/plant FW with little change in % DM (25.9 ± 0.2 to 27.7 ± 0.2) with increasing concentrations of NH_4NO_3 from 1-3 mM. Therefore, the DW of spinach leaves also increased with increased NH_4NO_3 supply from 1-3 mM.

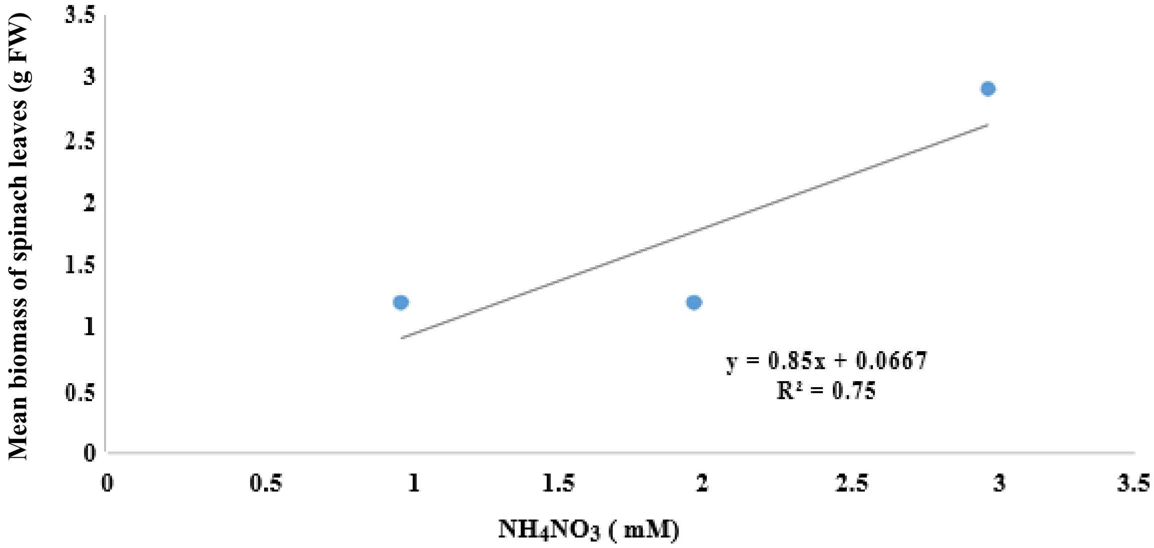

Figure 3.5 Biomass of spinach leaves (mean of ten plants FW) grown with different concentrations of NH_4NO_3 under greenhouse conditions.

There are several reports that have shown that crop plants fertilised with different concentrations of NO_3^- had better biomass and yield when compared to plants fertilised with NH_4^+ (Zhang *et al*., 2005; Montemurro *et al.,* 1998; Van Der Boon *et al.,* 1990). Biomass of spinach leaves produced at increasing concentrations of NO_3^- from 2-6 mM had higher growth rate when compared with the biomass of spinach leaves produced at increasing concentrations of NH_4NO_3 from 1-3 mM.

The total oxalate levels in spinach leaves decreased linearly from 3.6 ± 0.3 to 2.1 ± 0.0 g/100 g DM and soluble oxalate decreased linearly from 2.0 ± 0.0 to 1.4 ± 0.0 g/100 g FW with increasing concentration of NH_4NO_3 from 1- 3 mM (Fig 3.6 and Table 3.3). Total oxalate and soluble oxalate decreased linearly by 71% and 64% respectively with an increase in NH_4NO_3 concentrations from 1-3 mM (Fig 3.6). There was a negative correlation between growth and total and soluble oxalate concentrations from 1-3 mM NH_4NO_3. The percent soluble oxalate of total oxalate in spinach leaves ranged between 56 and 72 (Table 3.3). Overall, spinach leaves grown under NH_4NO_3 had similar oxalate levels when compared with spinach leaves of similar size grown under NO_3^-.

Table 3.3 Biomass and mean total, soluble and insoluble oxalate (g/100 g ± SE DM) (mean values of ten plants) grown with different NH_4NO_3 concentrations under greenhouse conditions. Values in brackets are % soluble oxalate of total oxalate.

Plants watered with NH_4NO_3 concentrations (mM)		Biomass produced after five weeks of growth (g/plant FW)	Oxalate (g/100 g DM)		
			Total	Soluble	Insoluble
1		1.2 ± 0.2	3.6 ± 0.3	2.0 ± 0.0 (56)	1.6 ± 0.3
2		1.2 ± 0.2	3.2 ± 0.2	2.3 ± 0.0 (72)	0.9 ± 0.2
3		2.9 ± 0.3	2.1 ± 0.0	1.4 ± 0.0 (67)	0.7 ± 0.0
Analysis of variance	**df**	**Biomass**	**Total**	**Soluble**	**Insoluble**
Fertiliser treatment	**2**	*	***	***	***

Significance: ***P < 0.001; *P < 0.05

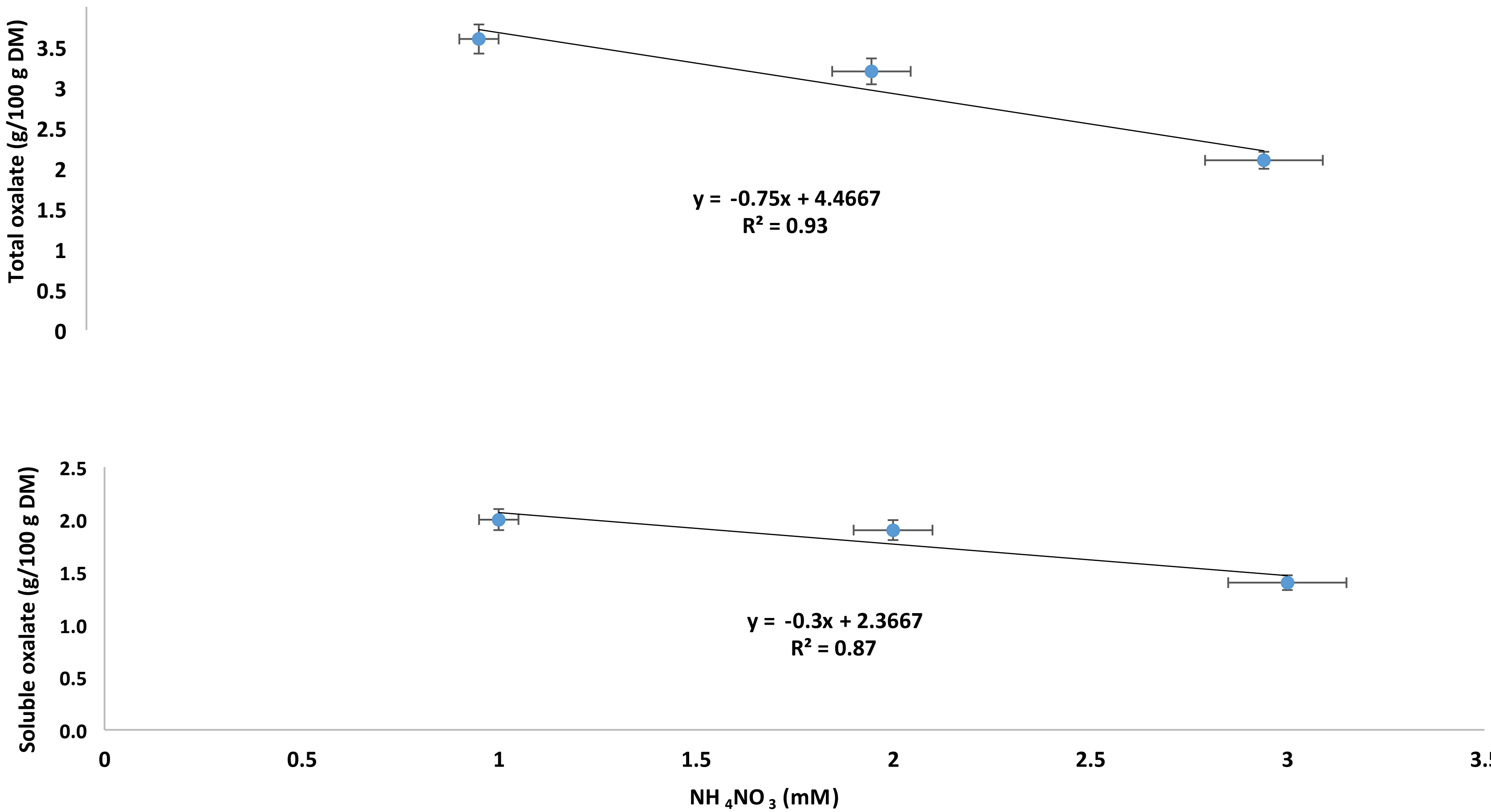

Figure 3.6 Total and soluble oxalate of spinach leaves (g/100 g DM) fertilised with different concentrations of NH_4NO_3 and grown under greenhouse conditions.

3.4 Conclusion

In conclusion, under greenhouse conditions the biomass of spinach leaves increased linearly with an increase in NO_3^- from 1-10 mM and NH_4NO_3 from 1-3 mM. With an increase in biomass, there was significant reduction in total and soluble oxalate levels per unit weight of spinach leaves with increasing nitrogen concentrations and form. The % DM changed little with increased NO_3^- and NH_4NO_3 supply and thus, leaf DW increased with increased NO_3^- or NH_4NO_3 supply. There is a negative correlation between leaf DW and leaf oxalate levels per unit DW.

Chapter 4

Growth and oxalate levels of spinach leaves fertilised with NO_3^- or NH_4NO_3 and grown under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

4.1 Introduction

Rising CO_2 levels not only have an impact on the environment but also affect plant functions such as growth, yield and nutritional quality (Giri *et al.,* 2016; Andrews *et al.,* 2019). Plants often respond positively to the increase in CO_2 levels by increasing growth (Jain *et al.,* 2007). Plants in their juvenile phase can grow exponentially, compared to mature plants. Atmospheric CO_2 concentration can also affect crop quality. Increasing the levels of CO_2 under controlled growth conditions can be beneficial for growing plant crops (Tremblay & Gosselin, 1998). Increased levels of CO_2 in the atmosphere can elevate plant growth by providing a 'fertilization' effect (Hamada *et al*., 2016). CO_2 enrichment of plants also increases water use efficiency and this subsequently improves growth (Andrews *et al.,* 2019).

Several studies have addressed the effect of elevated CO_2 on yield, biomass and nutrients in plants but very few on the effect it has on anti-nutritive components such as oxalates. Proietti *et al.* (2013) reported that there was a significant interaction between atmospheric CO_2 concentrations and the content of total oxalic acid. Increasing CO_2 concentration led to an increase in plant growth and a decrease in oxalic acid levels.

The present study focuses on the effect of elevated atmospheric CO_2 on growth and oxalate levels of spinach leaves fertilised with different concentrations of NO_3^- or NH_4NO_3 and grown in a controlled environment chamber.

4.2 Materials and Methods

Spinach plants *(S. oleracea.* L) were grown in one litre plant pots containing a standard N-free growing mixture (media: 1.3 L bark and 0.3 L pumice (Intelligro, Christchurch, New Zealand); fertilisers: 0-0-37 0.5 g Scotts Osmocote (Evergreen Garden Care, New South Wales, Australia), 1.7 g horticultural lime (Southern Horticultural Products Ltd, Christchurch, New Zealand), 0.5 g superphosphate, 0.5 g micromax and 1.7 g hydraflo (ICL Speciality Fertilizers, Tel Aviv, Israel) per pot containing two spinach plants) for five weeks under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber (Table 4.2) with temperature ranging from 15-25°C at Field Research Centre, Lincoln University, Canterbury, NZ (43° 38’ 43” S, 172° 27’ 43” E), 10 m above sea level. The seeds were sourced from Kings Seeds, Katikati, Bay of Plenty, New Zealand. Five spinach seeds were sown into each pot on the 3rd of August 2017 and once the spinach seeds had sprouted, the plants were reduced to two per pot on the 19th of August 2017. One group of spinach plants were fertilised with different concentrations of NO_3^- and another group of spinach plants were fertilised with different concentrations of NH_4NO_3 (Table 4.1). There were five replicate pots for each concentration of NO_3^- and NH_4NO_3. Each concentration of NO_3^- and NH_4NO_3 were made up to 2000 mL volume with tap water. Each pot was flushed through with 200 mL of the above solution twice a day (morning and evening).

Table 4.1 Nitrogen concentration and form, amount used (g/100 g), number of replicates and rate of dilution used in growing spinach plants under greenhouse conditions.

Nitrogen form	Concentrations (mM)	Amount added to each plant pot twice a day (g/100 g)	Replicates	Dilution
NO_3^-	1	0.20	Five pots per treatment	Each concentration made up to 2000 mL with tap water
	2	0.40		
	3	0.60		
	4	0.80		
	6	1.20		
	8	1.60		
	10	2.00		
NH_3NH_4	1	0.12	Five pots per treatment	Each concentration made up to 2000 mL with tap water
	2	0.24		
	3	0.32		

Table 4.2 Plant growth chamber parameters and range of values

Parameters	Range
Ambient CO_2 (ppm)	405 ± 4.1
Enriched CO_2 (ppm)	650 ± 3.2
Night temperature (°C)	15.0 ± 0.1
Day temperature (°C)	20.0 ± 0.2
Relative humidity (%)	64.0 ± 0.8
Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	1200 ± 0.0

4.2.1 Plant growth chamber

The BWD 40 series is a walk-in plant growth chamber (Conviron- Controlled Environments Ltd, Winnipeg, Canada). The standard lighting package for the BDW series chamber generates moderate to high-level light intensity and incorporates metal halide and halogen incandescent lamps. The BDW 40 series has a top-down airflow pattern which uniformly disperses conditioned air from the top of each plenum and returns the air through conditioning coils located at the bottom of the chamber area. Using an independent ventilation and conditioning system for the lamp loft helps to reduce heat transfer to the chamber area. Filtered and adjustable fresh air intake and exhaust openings enable effective air exchange to the chamber.

4.2.2 Harvesting

Spinach plants were harvested at the end of week five (9th September 2017). The whole plant was washed with cold tap water to remove soil particles and excess water was dabbed with a paper cloth.

4.2.3 Biomass and sample preparation

The whole plant weight of each spinach plant was recorded. The leaves, roots and stems of each spinach plant were weighed separately. Spinach leaves produced from each plant was used to determine the biomass. The leaves were then divided into three paper bags per NO_3^- or NH_4NO_3 concentration and dried. The dried leaves were ground into a fine powder using a mortar and pestle, and approximately 0.4 g of the powdered leaf sample was used for analysis.

4.2.4 Dry matter

The dry matter (DM) contents of spinach leaves were determined by drying in an oven (Watvic, Watson Victor Ltd., New Zealand) to a constant weight at 105°C for 24 hours, following a method outlined by Ruiz (2001).

4.2.5 Extraction of total and soluble oxalic acid

The measurement of total and soluble oxalates was performed following the method outlined by Savage *et al*. (2000). Three replicates of each sample (0.4 g per sample x three replicates) were extracted to measure total and soluble oxalate contents. 40 mL of 0.2 M HCL (Aristar, BDH Chemicals, Ltd., Poole, Dorset, UK) was added to volumetric flasks for the total oxalate extraction and also 40 mL of Nanopore II water (Barnstead International, Dubuque, Iowa, USA, 18 MΩ cm) was added for the extraction of soluble oxalates (Savage *et al.,* 2000). All flasks were placed in an 80°C shaking water bath for 20 minutes. The solutions were allowed to cool to 20°C and then made up to 100 mL with 0.2 M HCL for total oxalate and Nanopore II water for soluble oxalate, respectively.

4.2.6 Sample analysis

The extracts in the volumetric flasks were filtered through a cellulose acetate syringe filter with a pore size of 0.45 µm (dismic-25cs, Advantec, California, USA) into 1 mL glass HPLC vials. The samples were analysed with a high-performance liquid chromatography (HPLC) system, using a 300 mm x 7.8 mm Rezex ion exclusion column (Phenomenex Inc., Torrance, CA, USA) attached to a Cation-H guard column (Bio-Rad, Richmond, CA, USA) held at 25°C. The analysis was performed by injecting 20 µL of sample or standard onto the column using an aqueous solution of 25 mm sulphuric acid (HPLC grade Baker Chemicals, Phillipsburg NJ, USA) as the mobile phase, then pumped isocratically at 0.6 mL/min, with peaks detected at 210 nm. The HPLC equipment consisted of a Shimadzu LC-10AD pump, CTO-10A column oven, SPD-10Avp UV-Vis detector (Shimadzu, Kyoto, Japan) and a Waters 717 plus autosampler (Waters, Milford MA, USA). Data acquisition and processing were undertaken using the Peak Simple Chromatography Data System (model 203) and Peak Simple software version 4.37 (SRI Instruments, Torrance CA, USA). The oxalic acid peak was identified by comparing the retention time to a standard solution and by spiking an already-filtered sample containing a known amount of oxalic acid standard. The insoluble oxalate content of each sample was calculated by the difference between the total and the soluble oxalate contents (Holloway *et al.,* 1989).

4.2.7 Standard calibration

Two standard curves of oxalic acid (99.99% oxalic acid, Sigma-Aldrich Co., St. Louis, USA) were analysed, using standards of the following concentrations: 1, 2, 5, 10, 15 and 25 mg/100 mL. One batch of standards were prepared in 0.2 M HCL while the other was prepared in Nanopore II water. The acid standard curve was used for identifying and calculating the total oxalate content, while the water standard curve was used for the soluble oxalate content (Appendix 1). All blank and standard solutions were passed through a 0.45 µm cellulose acetate filter before analysis.

4.2.8 Statistical analysis

All calculations were performed using Excel 2016, and statistical analysis was carried out using Minitab Statistical Software (USA) (version 18.1) for Windows 10 (USA). A General Linear Model (GLM) was used to perform analysis of variance (ANOVA). All effects described as significant have a probability (P) value $P < 0.05$. A linear regression analysis was carried out on data obtained from experiments. The data from the results are presented as mean ± standard error (SE).

4.3 Results and Discussion

4.3.1 Biomass and oxalate levels of spinach leaves grown with different NO_3^- concentrations under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber

The biomass of spinach leaves grown under ambient CO2 conditions in a control environment chamber increased linearly from 2.6 ± 0.4 to 13.7 ± 0.7 g/plant FW and % DM increased from 23.9 ± 1.3 to 36.6 ± 1.7 with an increase in NO_3^- concentrations from 1- 10mM (Table 4.3 and Fig 4.1). The mean biomass (mean biomass of NO_3^- concentrations 1-10 mM) of spinach leaves increased by 64% when compared with biomass of spinach leaves grown under greenhouse conditions with an increase in NO_3^- concentration from 1-10 mM. Conditions in a greenhouse can vary considerably and physical factors such as light, temperature and humidity cannot be manipulated to maintain growth constancy. On the contrary, plant growth chambers allow for a better controlled environment (Measures *et al.,* 1973).

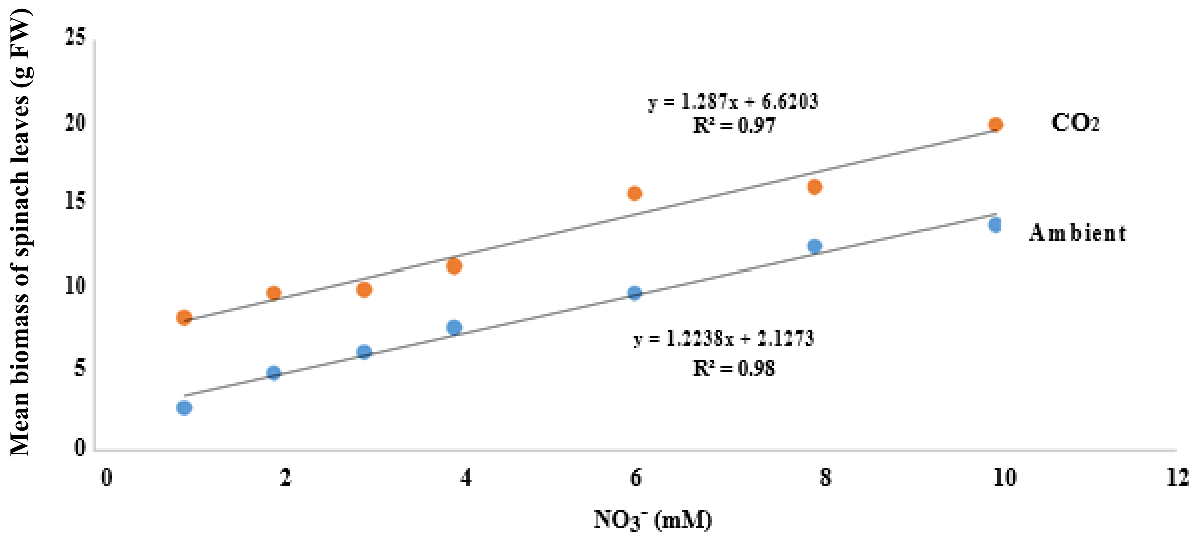

Figure 4.1 Biomass of spinach leaves (mean of ten plants FW) grown with different concentrations of NO_3^- under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

Under elevated atmospheric CO_2 conditions the biomass of spinach leaves increased linearly from 8.1 ± 0.6 to 19.8 ± 1.7 g/plant FW and % DM increased from 29.6 ± 1.7 to 35.7 ± 1.2 with an increase in NO_3^- concentration from 1-10 mM (Table 4.3 and Fig 4.1) The mean biomass (mean biomass of NO_3^- concentrations1-10 mM) of spinach leaves grown under elevated atmospheric CO_2 increased by 60% when compared to spinach leaves grown under ambient CO_2 conditions with an increase in NO_3^- concentration from 1-10 mM in a controlled environment chamber.

Table 4.3 Biomass of spinach leaves produced after five weeks of growth (mean value of ten plants) fertilised with different NO_3^- concentrations under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

Plants watered with NO_3^- concentrations (mM)	Biomass of spinach leaves produced after five weeks of growth (g/plant FW)	
	Ambient	Elevated atmospheric CO_2
1	2.6 ± 0.4	8.1 ± 0.6
2	4.7 ± 0.6	9.6 ± 0.4
3	6.0 ± 0.6	9.8 ± 1.5
4	7.5 ± 0.7	11.2 ± 0.7
6	9.6 ± 0.7	15.6 ± 1.1
8	12.4 ± 0.8	16.0 ± 0.8
10	13.7 ± 0.7	19.8 ± 1.7
Analysis of Variance	**df**	**Biomass**
Fertiliser treatment	6	***
Controlled environment conditions	1	***
Fertiliser treatment x controlled environment conditions	6	***

Significance: ***P < 0.001

The total oxalate levels in spinach leaves decreased linearly from 10.9 ± 0.1 to 5.1 ± 0.3 g/100 g DM and soluble oxalate levels decreased linearly from 6.5 ± 0.0 to 2.1 ± 0.0 g/100 g DM with an increase in NO_3^- concentrations from 1-10 mM grown under ambient conditions in a controlled environment chamber (Table 4.4). Total oxalate levels in spinach decreased linearly by 53% and soluble oxalate levels in spinach leaves decreased linearly by 68% with increased NO_3^- supply (Fig 4.2). The percent soluble oxalate of the total oxalate ranged between 41 and 68. In comparison, the total and soluble oxalate levels of spinach leaves grown under ambient conditions in a controlled environment chamber was similar to that of spinach leaves grown under greenhouse conditions with an increase in NO_3^- concentration from 1-10 mM.

Under elevated atmospheric CO_2 conditions, the total oxalate levels in spinach leaves decreased linearly from 6.8 ± 0.1 to 2.2 ± 0.3 g/100 g DM and soluble oxalate levels decreased linearly from 4.1 ± 0.1 to 1.3 ± 0.0 g/100 g DM with an increase in NO_3^- concentration from 1-10 mM (Table 4.4). The total and soluble oxalate levels of spinach leaves grown under elevated atmospheric CO_2 conditions reduced by 47% and 39% respectively, when compared to the total and soluble oxalate levels of spinach leaves grown under ambient conditions with an increase in NO_3^- concentration from 1-10 mM. The percent soluble oxalate of the total oxalate ranged between 59 and 60. Giri *et al.* (2016) stated that there was a decrease in the nutrient content of spinach plants grown under elevated atmospheric CO_2 due to dilution caused by high carbon accumulation. It is possible that, while increased levels of CO_2 decrease the levels of essential nutrients it also reduces anti-nutritive components such as oxalate

Table 4.4 Mean total, soluble and insoluble oxalate (g/100 g ± SE DM) of spinach leaves (mean value of ten plants) grown with different concentrations of NO_3^- under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber. Values in brackets are% soluble oxalate of total oxalate.

Plants watered with NO_3- concentrations (mM)	Oxalate (g/100 g DM) Total		Soluble		Insoluble	
	Ambient	Elevated atmospheric CO_2	Ambient	Elevated atmospheric CO_2	Ambient	Elevated atmospheric CO_2
1	10.9 ± 0.1	6.8 ± 0.1	6.5 ± 0.0 (60)	4.1 ± 0.1 (60)	4.4 ± 0.1	2.7 ± 0.0
2	8.6 ± 0.1	4.7 ± 0.2	5.3 ± 0.0 (62)	2.8 ± 0.1 (60)	3.3 ± 0.1	1.9 ± 0.1
3	7.8 ± 0.0	4.5 ± 0.1	5.3 ± 0.0 (68)	2.7 ± 0.0 (60)	2.5 ± 0.0	1.8 ± 0.1
4	7.2 ± 0.1	4.1 ± 0.2	4.8 ± 0.0 (67)	2.5 ± 0.1 (61)	2.4 ± 0.1	1.6 ± 0.1
6	6.9 ± 0.1	3.5 ± 0.1	4.6 ± 0.0 (67)	2.1 ± 0.1 (60)	2.3 ± 0.1	1.4 ± 0.0
8	6.2 ± 0.1	2.3 ± 0.3	4.2 ± 0.0 (68)	1.4 ± 0.0 (61)	2.0 ± 0.1	0.9 ± 0.3
10	5.1 ± 0.3	2.2 ± 0.3	2.1 ± 0.0 (41)	1.3 ± 0.0 (59)	3.0 ± 0.3	0.9 ± 0.3

Analysis of Variance	df	Total	Soluble	Insoluble
Fertiliser treatment	6	***	***	*
Controlled environment conditions	1	***	***	***
Fertiliser treatment x controlled environment conditions	6	ns	***	*

Significance: ns, not significant; ***P < 0.001; *P < 0.05

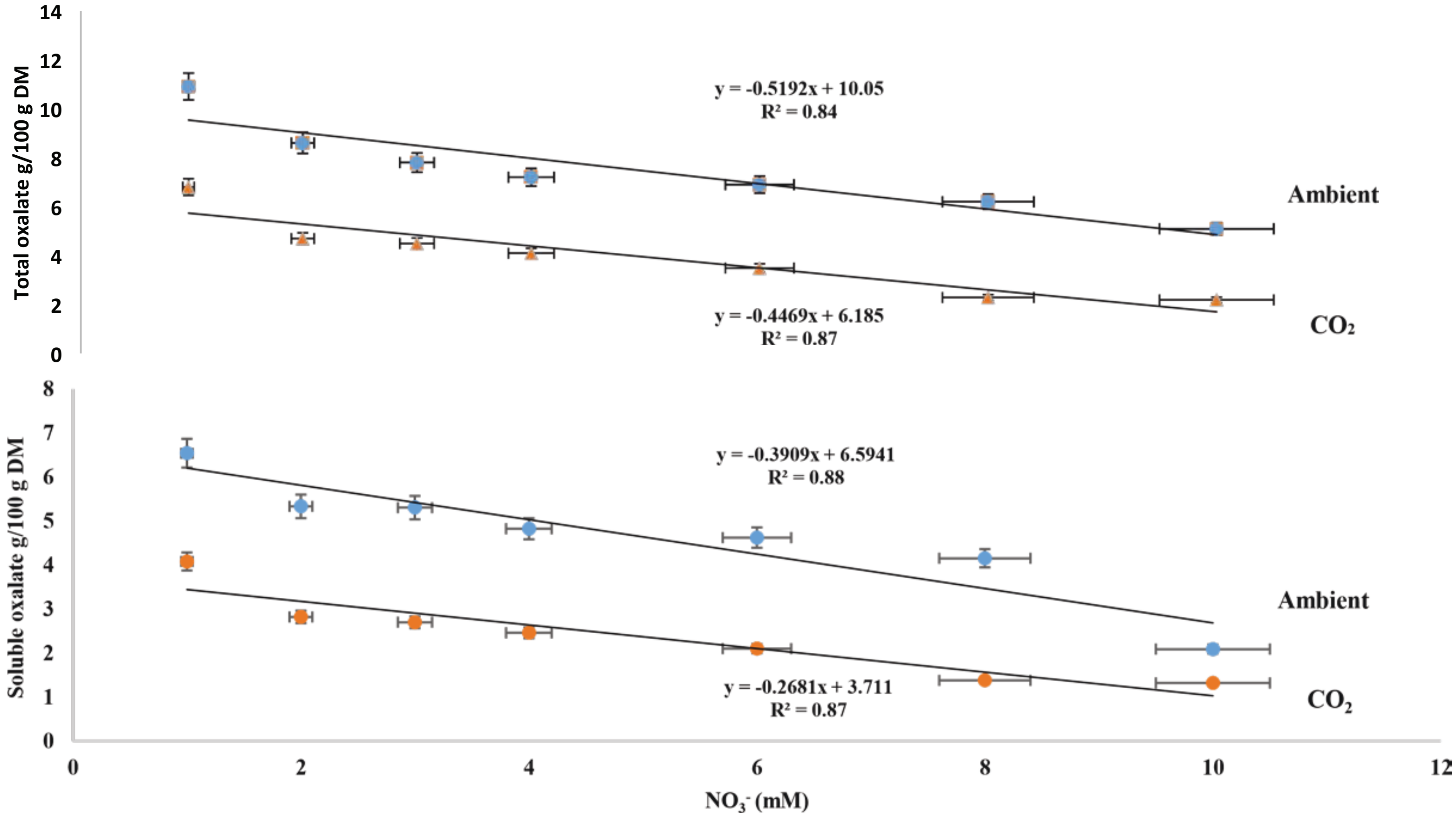

Figure 4.2 Total and soluble oxalate of spinach leaves (g/100 g DM) fertilised with different concentrations of NO3- and grown under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

4.3.2 Biomass and oxalate levels of spinach leaves grown with different NH_4NO_3 concentrations under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber

The biomass of spinach leaves grown under ambient CO_2 conditions in a controlled environment chamber increased linearly from 2.1 ± 0.3 to 3.8 ± 0.4 g/plant FW and % DM increased from 28.2 ± 0.2 to 32.3 ± 0.2 with an increase in NH_4NO_3 concentration from 1- 3 mM (Table 4.5 and Fig 4.3). Under elevated atmospheric CO_2 conditions the biomass of spinach leaves increased linearly from 7.3 ± 0.3 to 9.7 ± 1.0 g/plant FW and % DM increased from 28.8 ± 0.4 to 30.4 ± 0.6 with an increase in NH_4NO_3 concentration from 1-3 mM (Table 4.5 and Fig 4.3)

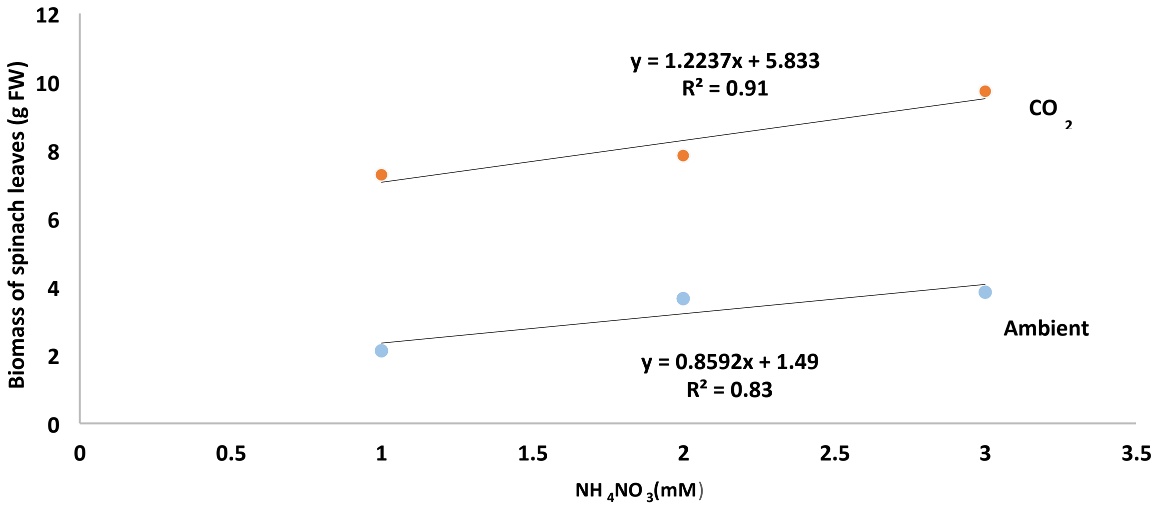

Figure 4.3 Biomass of spinach leaves (mean of ten plants FW) grown with different concentrations of NH_4NO_3 under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

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Table 4.5 Biomass of spinach leaves produced after five weeks of growth (g/100 g ± SE FW) (mean value of ten plants) grown with different NH_4NO_3 concentrations under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

Plants watered with NH_4NO_3 concentrations (mM)	Biomass of spinach leaves produced after five weeks of growth (g/plant FW)	
	Ambient	Elevated atmospheric CO_2
1	2.1 ± 0.3	7.3 ± 0.3
2	3.7 ± 0.3	7.7 ± 0.0
3	3.8 ± 0.4	9.7 ± 1.0
Analysis of Variance	**df**	**Biomass**
Fertiliser treatment	**2**	***
Controlled environment conditions	**1**	***
Fertiliser treatment x controlled environment conditions	**2**	*

Significance: ***P < 0.001; *P < 0.05

The total oxalate levels in spinach leaves grown under ambient conditions in a controlled environment chamber decreased linearly from 7.6 ± 0.3 to 5.4 ± 0.4 g/100 g DM and soluble oxalate levels decreased linearly from 5.8 ± 0.2 to 4.1 ± 0.0 g/100 g DM with increasing NH_4NO_3 concentrations from 1-3 mM (Table 4.6 and Fig 4.4). The total oxalate levels in spinach leaves decreased linearly by 41% with an increase in NH_4NO_3 supply (Fig 4.4). The percent soluble oxalate of the total oxalate ranged between 76 and 93. Overall, spinach leaves grown under NH_4NO_3 had similar oxalate levels when compared with spinach leaves of similar size grown under NO_3^-.

Under elevated atmospheric CO_2 conditions, the total oxalate levels in spinach leaves decreased linearly from 5.0 ± 0.1 to 2.8 ± 0.1 g/100 g DM and soluble oxalate levels reduced from 4.6 ± 0.1 to 2.3 ± 0.1 g/100 g DM with an increase in NH_4NO_3 concentration from 1-3 mM (Table 4.6 and Fig 4.4). The total oxalate levels in spinach leaves decreased linearly by 40% with an increase in NH_4NO_3 supply (Fig 4.4). The percent soluble oxalate of the total oxalate ranged between 81 and 92. Overall, the total and soluble oxalate levels of spinach leaves grown under elevated atmospheric CO_2 reduced by 42% and 36 % when compared to total and soluble oxalate levels of spinach leaves grown under ambient CO_2 conditions in a controlled environment chamber.

Table 4.6 Mean total, soluble and insoluble oxalate (g/100 g ± SE DM) of spinach leaves (mean value of ten plants) grown with different NH_4NO_3 concentrations under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber. Values in brackets are % soluble oxalate of total oxalate

Plants watered with NH_4NO_3 concentrations (mM)	Oxalate (g/100 g DM)					
	Total		Soluble		Insoluble	
	Ambient	Elevated atmospheric CO_2	Ambient	Elevated atmospheric CO_2	Ambient	Elevated atmospheric CO_2
1	7.6 ± 0.3	5.0 ± 0.1	5.8 ± 0.2 (76)	4.6 ± 0.1 (92)	1.8 ± 0.1	1.6 ± 0.0
2	5.7 ± 0.2	3.2 ± 0.1	5.3 ± 0.0 (93)	2.6 ± 0.0 (81)	0.4 ± 0.2	0.6 ± 0.1
3	5.4 ± 0.4	2.8 ± 0.1	4.1 ± 0.0 (78)	2.3 ± 0.1 (82)	1.3 ± 0.4	0.5 ± 0.0

Analysis of Variance	df	Total	Soluble	Insoluble
Fertiliser treatment	1	***	***	*
Controlled environment conditions	1	***	***	***
Fertiliser treatment x controlled environment conditions	2	ns	***	*

Significance: ns, not significant; ***P < 0.001; *P < 0.05

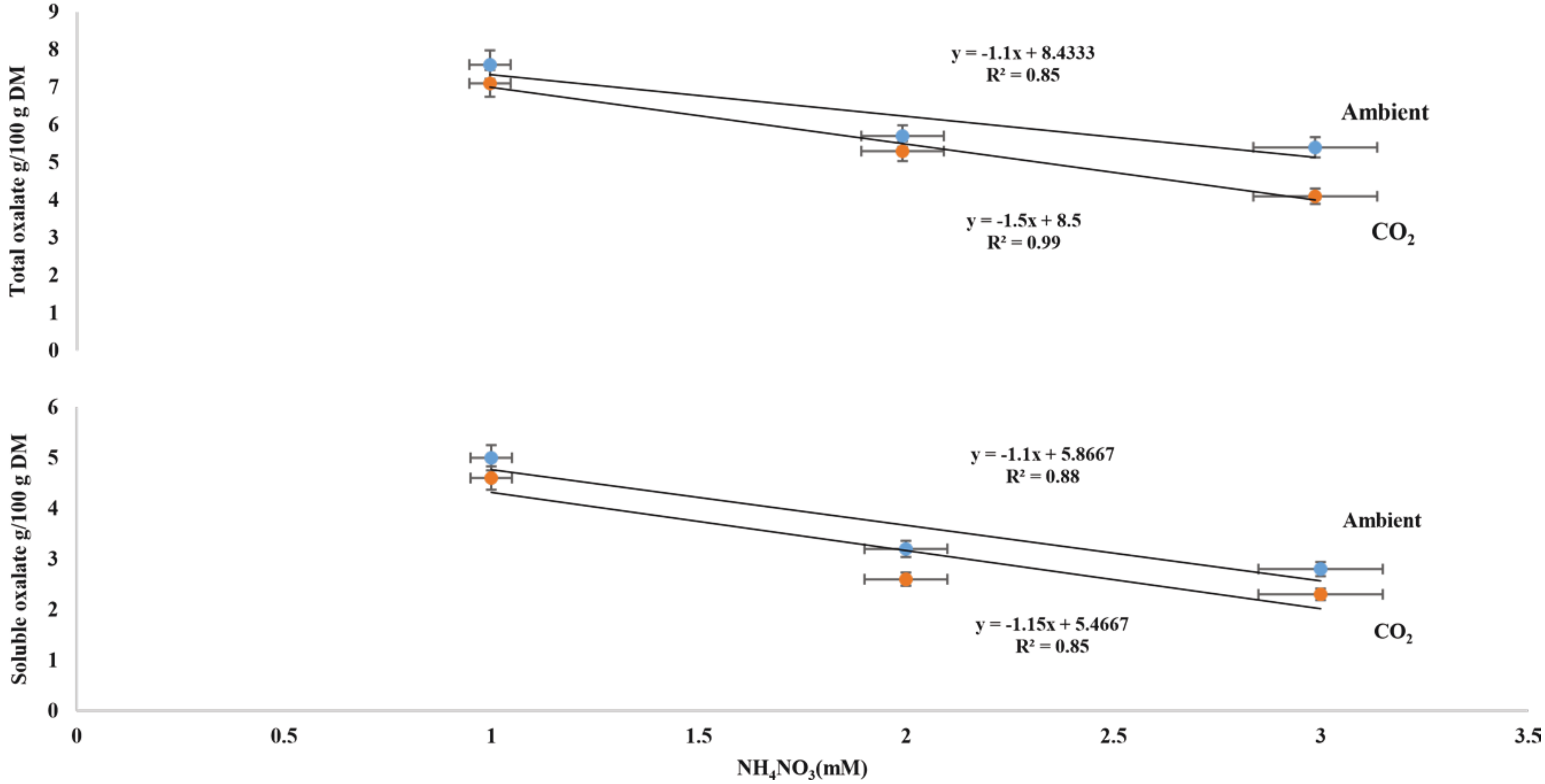

Figure 4.4 Total and soluble oxalate of spinach leaves (g/100 g DM) fertilised with different concentrations of NH_4NO_3 and grown under ambient and elevated atmospheric CO_2 conditions in a controlled environment chamber.

4.4 Conclusion

In conclusion, the biomass of spinach leaves grown under elevated atmospheric CO_2 conditions increased significantly in comparison to the biomass of spinach leaves grown under ambient CO_2 conditions in a controlled environment chamber with an increase in NO_3^- concentrations from 1-10 mM and NH_4NO_3 concentrations from 1-3 mM. There was a linear reduction of total and soluble oxalate levels in spinach leaves grown under elevated atmospheric CO_2 conditions with an increase in NO_3^- or NH_4NO_3 supply. These results show that there was a negative correlation between growth and total and soluble oxalate concentrations from 1- 10 mM NO_3^- and 1-3 mM NH_4NO_3 supply. Thus at elevated CO_2 total plant oxalate and leaf oxalate concentration both decreased with NO_3^- and NH_4 supply.

Chapter 5
Final Discussion and Conclusions

Worldwide spinach is an economically important vegetable crop and is considered one of the healthiest vegetables in the human diet. Spinach is a rich source of several minerals and micronutrients such as iron, calcium, potassium, vitamin A, K and C (Hedges & Lister 2007). However, spinach contains high levels of oxalates, an anti-nutritive component (Savage *et al.,* 2000; Morrison & Savage, 2003; Siener *et al.,* 2006; Savage & Mårtensson, 2010). Oxalate content in spinach varies with cultivar and environmental conditions and can range between 53.4 and 12,576.2 mg/100 g DM (Radek & Savage 2008; Mou, 2008).

Oxalic acid occurs as a product of metabolism in plant tissues and is found in the form of soluble or insoluble oxalate. The two major effects of consuming high oxalate containing foods are; firstly, oxalic acid can form insoluble salts by binding to minerals such as calcium, magnesium and iron in the foods and, thereby, decrease the bioavailability of many such minerals that are essential for the human body (Noonan & Savage, 1999). The insoluble oxalate salts that are not absorbed are excreted in the faeces (Marengo & Romani, 2008). Secondly, soluble oxalate, once absorbed, cannot be used in the body and needs to be excreted. In the process of removing soluble oxalate from venous blood being filtered by the kidneys, soluble oxalates may become insoluble oxalate by being bound to calcium. This accumulation of calcium oxalate in the kidneys can lead to the formation of kidney stone (Williams & Wandzilak, 1989; Noonan & Savage, 1999; Chai & Liebman, 2005).

The main objectives of the first experiment (chapter 3) were to determine the effects of nitrogen concentration and form (NO_3^- and NH_4^+) on growth and oxalate levels in spinach leaves grown under greenhouse conditions (ambient CO_2). The aim of the second experiment (controlled environment chamber; chapter 4) was to evaluate the effect of elevated CO_2 concentration on growth and oxalate levels in spinach leaves fertilised with different concentrations of NO_3^- or NH_4NO_3.

Under greenhouse conditions biomass of spinach leaves increased linearly by 6-fold while total oxalate levels reduced from 9.2 ± 0.4 to 3.3 ± 0.1 g/100 g DM and soluble oxalate levels reduced from 4.7 ± 0.1 to 2.3 ± 0.0 g/100 g DM with increasing concentrations of NO_3^- from 1-10 mM. Spinach plants fertilised with NH_4NO_3 also showed similar effect. The biomass increased linearly by 2.5-fold while total oxalate levels reduced from 3.6 ± 0.3 to 2.1 ± 0.0 g/100 g DM and soluble oxalate levels reduced from 2.0 ± 0.0 to 1.4 ± 0.0 g/100 g DM with increasing NH_4NO_3

concentrations from 1-3 mM. Overall, spinach leaves grown under NH_4NO_3 had similar oxalate levels when compared with spinach leaves of similar size grown under NO_3^-. Overall, the proportional increase in growth was greater than the proportional decrease in oxalate levels across all treatments. As a result, total plant oxalate increased but the leaf concentration decreased with increased growth across all treatments.

Under controlled environment conditions, biomass of spinach leaves grown under ambient conditions increased linearly by 5.3-fold while total oxalate levels decreased from 10.9 ± 0.1 to 5.1 ± 0.3 g/100 g DM and soluble oxalate levels decreased from 6.5 ± 0.0 to 2.1 ± 0.0 g/100 g DM with an increase in NO_3^- concentrations from 1-10 mM. The biomass increased linearly by 1.8-fold while the total oxalate levels decreased from 7.6 ± 0.3 to 5.4 ± 0.4 g/100 g DM and soluble oxalate levels decreased from 5.8 ± 0.2 to 4.1 ± 0.0 g/100 g DM with increasing NH_4NO_3 concentrations from 1-3 mM. Overall, the results obtained under different NO_3^- and NH_4NO_3 concentrations at ambient CO_2 in controlled environment conditions were similar to those obtained under greenhouse conditions. Under elevated atmospheric CO_2 conditions, biomass of spinach leaves increased linearly by 2.5-fold while total oxalate levels decreased from 6.8 ± 0.1 to 2.2 ± 0.3 g/100 g DM and soluble oxalate levels decreased from 4.1 ± 0.1 to 1.3 ± 0.0 g/100 g DM with an increase in NO_3^- concentrations from 1-10 mM. The biomass increased linearly by 1.3-fold while the total oxalate levels decreased from 5.0 ± 0.1 to 2.8 ± 0.1 g/100 g DM and soluble oxalate levels decreased from 4.6 ± 0.1 to 2.3 ± 0.1 g/100 g DM with increasing NH_4NO_3 concentrations from 1-3 mM. Overall, the proportional increase in growth was greater than the proportional decrease in oxalate levels. As a result total plant oxalate increased but the leaf concentration decreased with different NO_3^- and NH_4NO_3.

In conclusion, with increasing concentration of nitrogen (NO_3^- and NH_4NO_3) there was an increase in the overall biomass and yield of spinach leaves and an overall reduction in the total and soluble oxalate levels of spinach leaves grown under greenhouse conditions. In addition, elevated atmospheric CO_2 gave a further increase in growth across all N concentrations and a further decrease in total and soluble oxalate levels across all N concentrations. Although elevated CO_2 is known to reduce the concentration of essential nutrients such as calcium and iron in spinach plants, it can be beneficial as it reduces the concentration of oxalates which is known to cause kidney stone formation in humans.

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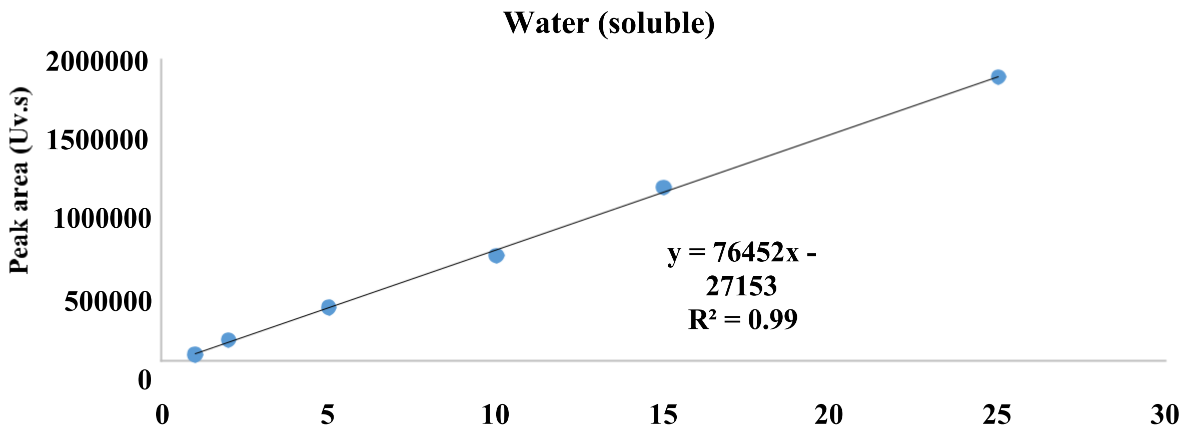
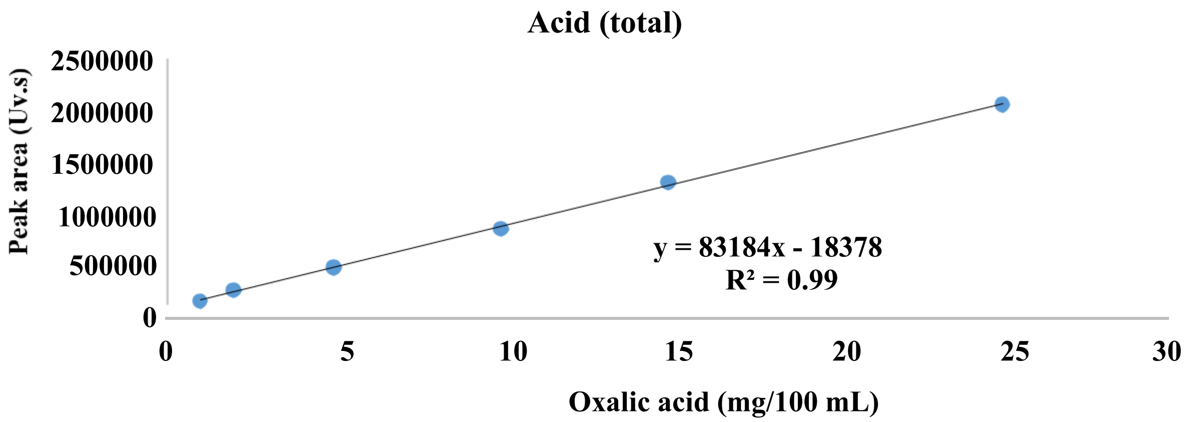
Appendix A :

Total and soluble oxalic acid standard curves.

Concentration of oxalic acid (mg/100 mL)	Peak areas (Uv.s)	
	Water	Acid
1	44994	55359
2	141288	162104
5	355540	394622
10	699529	788752
15	1150099	1262497
25	1879826	2051070





Appendix B :

Mean biomass and % DM of spinach leaves (mean weight of ten plants) fertilised with different concentrations of NO_3^- and grown under greenhouse conditions.

NO_3^- concentrations (mM)	Biomass of spinach leaves (g/plant FW)	Mean biomass (g/plant FW)	SE	Spinach leaves (g DM)	% DM	Mean % DM	SE
1	1.12	1.5	0.3	0.80	29	27.1	0.2
	1.03			0.68	34		
	2.09			1.36	35		
	3.11			2.46	21		
	1.11			0.87	22		
	1.89			1.34	29		
	2.14			1.56	27		
	0.99			0.80	19		
	0.87			0.62	29		
	0.27			0.20	26		
2	2.21	2.0	0.2	1.48	33	29.3	0.2
	2.08			1.41	32		
	2.78			2.06	26		
	1.96			1.47	25		
	1.41			1.00	29		
	2.01			0.99	32		
	1.77			1.29	27		
	1.36			0.94	31		
	1.25			0.88	30		
	3.41			2.46	28		
3	3.01	3.1	0.3	2.14	29	29.5	0.2
	1.78			1.39	22		
	2.99			2.42	19		
	2.87			2.07	28		
	1.54			0.99	36		
	3.68			2.39	35		
	3.97			2.78	30		
	4.01			2.77	31		
	3.98			2.79	30		
	3.14			2.04	35		
4	3.89	4.3	0.4	2.72	30	29.6	0.2
	3.47			2.60	25		
	3.55			2.52	29		
	2.14			1.52	29		
	5.66			3.68	35		
	5.78			3.81	34		
	5.14			3.60	30		
	5.21			3.70	29		
	4.18			3.18	24		
	4.44			3.06	31		
6	3.44	6.2	0.5	2.44	29	31.1	0.3
	6.47			4.40	32		
	7.98			5.51	31		
	7.74			4.64	40		

	6.44			4.57	29		
	7.14			4.93	31		
	6.32			4.30	32		
	4.11			2.88	30		
	5.78			4.10	29		
	5.99			4.31	28		
8	7.77	8.1	0.5	5.67	27	27.0	0.4
	8.41			5.97	29		
	8.98			6.11	32		
	8.36			6.02	28		
	10.57			8.03	24		
	9.78			7.63	22		
	5.14			4.06	21		
	7.47			5.45	27		
	6.47			4.46	31		
	7.11			5.05	29		
10	10.23	9.2	0.4	7.16	30	31.4	0.3
	10.58			6.98	34		
	10.54			7.27	31		
	8.99			6.11	32		
	9.36			6.65	29		
	6.17			4.32	30		
	8.66			5.37	38		
	9.14			5.85	36		
	9.24			6.65	28		
	8.66			6.41	26		

Appendix C :

Mean total and soluble oxalate levels of spinach leaves fertilised with different concentrations of NO_3^- and grown under greenhouse conditions.

Extraction	NO_3^- concentration (mM)	Replicates	Sample weight (g)	Peak Area (Uv.s)	Oxalic acid (mg/100mL)	Oxalic acid (mg/g)	Oxalic acid (mg/100 g)	Oxalic acid (g/100 g)	Mean oxalic acid (g/100 g)	SE
Acid (total)	1	1	0.441	2900639	38.79	87.91	8791.25	8.8	9.2	0.4
		2	0.428	3061810	40.94	95.63	9563.33	9.6		
		3	0.401	2812109	37.60	93.73	9372.89	9.4		
	2	1	0.402	2669781	35.70	88.88	8887.77	8.9	8.4	0.5
		2	0.431	2756863	36.87	85.56	8555.54	8.6		
		3	0.441	2589959	34.64	78.52	7852.09	7.9		
	3	1	0.416	2001947	26.78	64.32	6432.46	6.4	6.4	0.1
		2	0.428	2100158	28.09	65.62	6561.71	6.6		
		3	0.422	1994019	26.67	63.22	6321.97	6.3		
	4	1	0.453	1769699	23.67	52.22	5221.65	5.2	5.2	0.1
		2	0.457	1723480	23.06	50.49	5048.69	5.0		
		3	0.451	1792908	23.99	53.15	5314.66	5.3		
	6	1	0.461	1754190	23.47	50.96	5096.14	5.1	5.1	0.1
		2	0.451	1699472	22.74	50.37	5036.90	5.0		
		3	0.461	1803891	24.13	52.32	5232.40	5.2		
	8	1	0.451	1566201	20.96	46.49	4648.57	4.6	4.5	0.2
		2	0.462	1604874	21.47	46.49	4648.73	4.6		
		3	0.499	1615921	21.62	43.34	4333.56	4.3		
	10	1	0.468	1131553	15.15	32.36	3236.02	3.2	3.3	0.1
		2	0.464	1191019	15.94	34.39	3438.83	3.4		
		3	0.469	1171100	15.68	33.43	3343.20	3.3		
Water (soluble)	1	1	0.459	1661765	22.23	48.45	4844.78	4.8	4.7	0.1
		2	0.460	1601098	21.42	46.56	4655.95	4.7		
		3	0.462	1614789	21.60	46.75	4675.39	4.7		
	2	1	0.458	1381765	18.49	40.38	4038.27	4.0	4.1	0.1
		2	0.451	1401098	18.75	41.60	4160.10	4.2		
		3	0.450	1394789	18.67	41.49	4148.76	4.1		
	3	1	0.458	1002764	13.43	29.33	2932.91	2.9	3.0	0.1
		2	0.460	1102167	14.76	32.06	3205.55	3.2		
		3	0.453	1010215	13.53	29.89	2989.27	3.0		
	4	1	0.451	1111553	14.88	33.00	3300.19	3.3	3.2	0.1
		2	0.463	1091019	14.61	31.57	3156.73	3.2		
		3	0.464	1081100	14.47	31.23	3122.68	3.1		
	6	1	0.434	992764	13.29	30.62	3062.26	3.1	3.0	0.1
		2	0.429	1001669	13.41	31.26	3126.40	3.1		
		3	0.456	999872	13.39	29.35	2935.37	2.9		
	8	1	0.451	799822	11.44	25.36	2536.01	2.5	2.5	0.0
		2	0.453	804987	11.51	25.39	2539.38	2.5		
		3	0.442	787067	11.27	25.50	2550.50	2.6		
	10	1	0.447	683236	9.89	22.13	2212.61	2.2	2.2	0.0
		2	0.442	701069	10.13	22.94	2294.28	2.3		
		3	0.453	698718	10.10	22.32	2231.61	2.2		

Appendix D :

Mean biomass and % DM of spinach leaves (mean weight of ten plants) fertilised with different concentrations of NH_4NO_3 and grown under greenhouse conditions.

NH_4NO_3 concentration (mM)	Biomass of spinach leaves (g/plant FW)	Mean biomass (g/plant FW)	SE	Spinach leaves (g DM)	% DM	Mean % DM	SE
1	0.56	1.2	0.2	0.40	29	27.7	0.2
	0.99			0.66	33		
	1.09			0.85	22		
	1.05			0.74	30		
	1.78			1.32	26		
	1.66			1.25	25		
	1.21			0.87	28		
	2.89			1.99	31		
	0.64			0.47	27		
	0.44			0.33	26		
2	1.54	1.2	0.2	1.19	23	25.9	0.2
	0.11			0.07	35		
	0.67			0.53	21		
	1.98			1.60	19		
	1.74			1.29	26		
	1.59			1.19	25		
	0.86			0.41	29		
	2.47			1.93	22		
	0.84			0.60	29		
	0.66			0.46	30		
3	3.41	2.9	0.3	2.59	24	27.3	0.2
	3.98			2.71	32		
	3.73			2.61	30		
	1.54			1.08	30		
	2.87			1.95	32		
	2.45			1.84	25		
	3.69			2.77	25		
	2.48			1.76	29		
	1.97			1.44	27		
	1.69			1.37	19		

Appendix E :

Mean total and soluble oxalate levels of spinach leaves fertilised with different concentrations of NH_4NO_3 and grown under greenhouse conditions.

Extraction	NH_4NO_3 concentration (mM)	Replicates	Sample weight (g)	Peak Area (Uv.s)	Oxalic acid (mg/100mL)	Oxalic acid (mg/g)	Oxalic acid (mg/100 g)	Oxalic acid (g/100 g)	Mean oxalic acid (g/100 g)	SE
Acid (total)	1	1	0.413	1205789	16.14	39.12	3911.72	3.9	3.6	0.3
		2	0.448	1131465	15.15	33.82	3382.45	3.4		
		3	0.436	1179897	15.79	36.22	3621.60	3.6		
	2	1	0.443	1005178	13.46	30.42	3041.61	3.0	3.2	0.2
		2	0.440	1098324	14.70	33.42	3341.77	3.3		
		3	0.442	1056147	14.14	32.01	3201.31	3.2		
	3	1	0.433	688433	9.23	21.33	2133.33	2.1	2.1	0
		2	0.431	659746	8.84	20.51	2051.34	2.1		
		3	0.440	691941	9.27	21.10	2109.54	2.1		
Water (soluble)	1	1	0.432	629987	8.45	19.57	1956.83	2.0	2	0
		2	0.432	631428	8.46	19.60	1960.39	2.0		
		3	0.422	627434	8.41	19.92	1992.32	2.0		
	2	1	0.441	608741	8.16	18.51	1850.75	1.9	1.9	0
		2	0.440	597369	8.01	18.20	1820.42	1.8		
		3	0.432	611247	8.20	18.97	1896.62	1.9		
	3	1	0.428	461612	6.20	14.48	1448.29	1.5	1.4	0
		2	0.445	485763	6.52	14.65	1464.51	1.5		
		3	0.444	467766	6.28	14.15	1415.25	1.4		

Appendix F :

Mean biomass and % DM of spinach leaves (mean weight of ten plants) fertilised with different concentrations of NO_3^- and grown under ambient CO_2 conditions in a controlled environment chamber.

NO_3^- concentrations (mM)	Biomass of spinach leaves (g/plant FW)	Mean biomass (g/plant FW)	SE	Spinach leaves (g DM)	% DM	Mean % DM	SE
1	1.62	2.6	0.4	1.12	31	23.9	1.3
	2.08			1.62	22		
	2.79			2.09	25		
	2.65			2.12	20		
	1.86			1.51	19		
	1.96			1.51	23		
	3.46			2.53	27		
	0.74			0.59	20		
	4.89			3.47	29		
	3.82			2.94	23		
2	7.01	4.7	0.6	5.12	27	25.2	1.2
	3.78			2.80	26		
	7.11			5.40	24		
	4.34			3.39	22		
	2.96			2.25	24		
	1.09			0.88	19		
	6.15			4.67	24		
	4.33			3.07	29		
	5.55			3.77	32		
3	6.55	6.0	0.6	5.17	21	28.1	2.1
	6.54			4.12	37		
	5.54			4.49	19		
	8.21			5.83	29		
	6.48			4.99	23		
	9.01			6.85	24		
	3.78			2.34	38		
	4.02			2.65	34		
	3.91			3.01	23		
	5.08			3.40	33		
4	5.37	7.5	0.7	3.81	29	29.6	1.6
	8.85			6.02	32		
	8.24			5.27	36		
	7.70			6.01	22		
	6.33			4.37	31		
	7.16			5.23	27		
	7.38			5.46	26		
	6.41			4.17	35		
	9.86			6.70	32		
	6.05			4.48	26		
	12.98			10.12	22		
	8.59			6.10	29		
	8.83			5.56	37		

6	7.64	9.6	0.7	5.27	31	33.4	2.7
	7.48			5.01	33		
	14.67			10.27	30		
	8.78			5.97	32		
	10.56			6.76	36		
	6.76			2.91	57		
	11.29			8.24	27		
8	10.48	12.4	0.8	6.92	34	35.6	1.5
	9.24			5.54	40		
	9.46			6.43	32		
	12.68			8.75	31		
	13.65			8.19	40		
	16.21			10.37	36		
	13.53			8.25	39		
	12.8			8.32	35		
	13.13			8.01	39		
	7.41			5.19	30		
10	12.79	13.7	0.7	7.03	45	36.6	1.7
	15.4			8.78	43		
	12.29			8.23	33		
	13.98			8.81	37		
	13.50			8.10	40		
	13.16			9.34	29		
	12.73			8.91	30		
	12.75			9.05	29		
	17.00			10.03	41		
	8.76			5.34	39		

Appendix G :

Mean total and soluble oxalate levels of spinach leaves fertilised with different concentrations of NO_3^- and grown under ambient CO_2 in a controlled environment chamber.

Extraction	NO_3 concentration (mM)	Replicates	Sample weight (g)	Peak Area (Uv.s)	Oxalic acid (mg/100mL)	Oxalic acid (mg/g)	Oxalic acid (mg/100 g)	Oxalic acid (g/100 g)	Mean oxalic acid (g/100 g)	SE
Acid (total)	1	1	0.402	892021	44.0	109.5	10950.4	11.0	10.9	0.1
		2	0.405	896022	44.2	109.2	10923.1	10.9		
		3	0.403	880023	43.4	107.6	10761.2	10.8		
	2	1	0.404	710465	34.1	84.5	8450.4	8.5	8.6	0.1
		2	0.404	729456	35.2	87.1	8706.2	8.7		
		3	0.400	719039	34.6	86.5	8651.6	8.7		
	3	1	0.405	659120	31.3	77.4	7739.5	7.7	7.8	0.0
		2	0.403	667046	31.8	78.8	7885.0	7.9		
		3	0.401	661098	31.5	78.4	7843.6	7.8		
	4	1	0.406	620671	29.3	72.1	7205.1	7.2	7.2	0.1
		2	0.406	609298	28.6	70.5	7052.6	7.1		
		3	0.400	618878	29.2	72.9	7288.8	7.3		
	6	1	0.403	588900	27.5	68.3	6829.6	6.8	6.9	0.1
		2	0.405	593401	27.8	68.6	6856.4	6.9		
		3	0.402	602190	28.2	70.3	7026.6	7.0		
	8	1	0.406	561042	26.0	64.1	6405.7	6.4	6.2	0.1
		2	0.403	521100	23.8	59.1	5914.0	5.9		
		3	0.405	549870	25.4	62.7	6271.4	6.3		
	10	1	0.401	487045	22.0	54.8	5481.3	5.5	5.1	0.3
		2	0.400	422098	18.4	46.1	4611.3	4.6		
		3	0.406	479999	21.6	53.2	5319.3	5.3		
Water (soluble)	1	1	0.405	640089	26.4	65.2	6515.8	6.5	6.5	0.0
		2	0.406	641408	26.4	65.1	6511.7	6.5		
		3	0.401	630284	26.0	64.9	6490.8	6.5		
	2	1	0.401	500011	21.2	52.9	5294.3	5.3	5.3	0.0
		2	0.400	511014	21.6	54.1	5408.8	5.4		
		3	0.405	502100	21.3	52.6	5261.0	5.3		
	3	1	0.406	499980	21.2	52.3	5228.8	5.2	5.3	0.0
		2	0.402	511900	21.7	53.9	5390.1	5.4		
		3	0.401	501098	21.3	53.0	5304.3	5.3		
	4	1	0.400	452690	19.5	48.7	4871.9	4.9	4.8	0.0
		2	0.405	459138	19.7	48.7	4870.3	4.9		
		3	0.404	450123	19.4	48.0	4800.2	4.8		
	6	1	0.400	423341	18.4	46.0	4601.6	4.6	4.6	0.0
		2	0.401	436987	18.9	47.2	4715.5	4.7		
		3	0.406	430021	18.7	45.9	4594.2	4.6		
	8	1	0.403	380901	16.8	41.8	4179.5	4.2	4.2	0.0
		2	0.402	389645	17.2	42.7	4270.0	4.3		
		3	0.405	391098	17.2	42.5	4251.6	4.3		
	10	1	0.401	160870	8.7	21.8	2179.6	2.2	2.1	0.0
		2	0.406	160542	8.7	21.5	2149.8	2.2		
		3	0.403	151236	8.4	20.8	2080.7	2.1		

Appendix H :

Mean biomass and % DM of spinach leaves (mean weight of ten plants) fertilised with different concentrations of NO_3^- and grown under elevated atmospheric CO_2 conditions in a controlled environment chamber.

NO_3- concentrations (mM)	Biomass of spinach leaves (g/plant FW)	Mean biomass (g/plant FW)	SE	Spinach leaves (g DM)	% DM	Mean % DM	SE
1	7.42	8.1	0.6	4.45	40	29.6	1.7
	11.19			7.27	35		
	5.03			3.47	31		
	9.92			7.14	28		
	5.89			4.42	24		
	8.23			6.09	26		
	9.52			7.24	24		
	9.02			6.95	23		
	6.81			4.70	31		
	7.69			5.08	34		
2	9.79	9.6	0.4	7.24	26	29.9	1.7
	10.49			6.92	34		
	11.44			7.89	31		
	10.27			6.37	38		
	9.03			5.42	31		
	8.44			5.99	29		
	9.63			6.84	22		
	11.55			7.85	32		
	7.11			4.26	34		
	8.53			6.65	22		
3	7.64	9.8	1.5	5.96	22	30.4	1.4
	3.18			2.13	33		
	9.75			6.24	36		
	10.96			8.11	26		
	5.88			4.59	33		
	18.72			13.29	29		
	10.71			7.71	28		
	8.89			6.40	35		
	15.90			10.97	31		
	6.05			4.17	31		
4	11.55	11.2	0.7	7.05	39	30.5	1.8
	13.66			8.20	26		
	12.25			8.82	25		
	14.21			10.94	23		
	11.63			8.72	32		
	6.30			4.03	36		
	11.13			7.57	32		
	9.37			6.28	33		
	12.01			8.41	30		
	10.35			7.35	29		
	15.26			10.99	36		
	14.89			10.27	31		

6	15.71	15.6	1.1	12.41	31	31.0	1.3
	17.53			14.2	34		
	18.29			12.62	31		
	15.15			11.06	27		
	19.87			14.51	27		
	6.43			4.82	25		
	15.63			12.19	40		
	17.38			12.51	28		
8	12.06	16.0	0.8	8.08	33	34.7	1.6
	19.5			12.68	35		
	17.95			12.74	29		
	16.74			12.39	26		
	14.93			9.70	35		
	12.08			7.73	36		
	18.26			11.5	37		
	17.91			13.07	27		
	16.53			12.4	25		
	13.65			10.37	24		
10	25.37	19.8	1.7	20.04	21	35.7	1.2
	23.45			16.42	30		
	18.87			13.40	29		
	11.63			8.26	29		
	24.69			17.04	31		
	12.23			8.19	33		
	19.45			12.64	35		
	16.05			11.24	30		
	26.24			18.63	29		
	19.84			14.48	27		

Appendix I :

Mean total and soluble oxalate levels of spinach leaves fertilised with different concentrations of NO_3^- and grown under elevated atmospheric CO_2 conditions in a controlled environment chamber.

Extraction	NO_3 concentration (mM)	Replicates	Sample weight (g)	Peak Area (Uv.s)	Oxalic acid (mg/100mL)	Oxalic acid (mg/g)	Oxalic acid (mg/100 g)	Oxalic acid (g/100 g)	Mean oxalic acid (g/100 g)	SE
Acid (total)	1	1	0.402	892021	44.0	109.5	10950.4	11.0	10.9	0.1
		2	0.405	896022	44.2	109.2	10923.1	10.9		
		3	0.403	880023	43.4	107.6	10761.2	10.8		
	2	1	0.404	710465	34.1	84.5	8450.4	8.5	8.6	0.1
		2	0.404	729456	35.2	87.1	8706.2	8.7		
		3	0.400	719039	34.6	86.5	8651.6	8.7		
	3	1	0.405	659120	31.3	77.4	7739.5	7.7	7.8	0.0
		2	0.403	667046	31.8	78.8	7885.0	7.9		
		3	0.401	661098	31.5	78.4	7843.6	7.8		
	4	1	0.406	620671	29.3	72.1	7205.1	7.2	7.2	0.1
		2	0.406	609298	28.6	70.5	7052.6	7.1		
		3	0.400	618878	29.2	72.9	7288.8	7.3		
	6	1	0.403	588900	27.5	68.3	6829.6	6.8	6.9	0.1
		2	0.405	593401	27.8	68.6	6856.4	6.9		
		3	0.402	602190	28.2	70.3	7026.6	7.0		
	8	1	0.406	561042	26.0	64.1	6405.7	6.4	6.2	0.1
		2	0.403	521100	23.8	59.1	5914.0	5.9		
		3	0.405	549870	25.4	62.7	6271.4	6.3		
	10	1	0.401	487045	22.0	54.8	5481.3	5.5	5.1	0.3
		2	0.400	422098	18.4	46.1	4611.3	4.6		
		3	0.406	479999	21.6	53.2	5319.3	5.3		
Water (soluble)	1	1	0.405	640089	26.4	65.2	6515.8	6.5	6.5	0.0
		2	0.406	641408	26.4	65.1	6511.7	6.5		
		3	0.401	630284	26.0	64.9	6490.8	6.5		
	2	1	0.401	500011	21.2	52.9	5294.3	5.3	5.3	0.0
		2	0.400	511014	21.6	54.1	5408.8	5.4		
		3	0.405	502100	21.3	52.6	5261.0	5.3		
	3	1	0.406	499980	21.2	52.3	5228.8	5.2	5.3	0.0
		2	0.402	511900	21.7	53.9	5390.1	5.4		
		3	0.401	501098	21.3	53.0	5304.3	5.3		
	4	1	0.400	452690	19.5	48.7	4871.9	4.9	4.8	0.0
		2	0.405	459138	19.7	48.7	4870.3	4.9		
		3	0.404	450123	19.4	48.0	4800.2	4.8		
	6	1	0.400	423341	18.4	46.0	4601.6	4.6	4.6	0.0
		2	0.401	436987	18.9	47.2	4715.5	4.7		
		3	0.406	430021	18.7	45.9	4594.2	4.6		
	8	1	0.403	380901	16.8	41.8	4179.5	4.2	4.2	0.0
		2	0.402	389645	17.2	42.7	4270.0	4.3		
		3	0.405	391098	17.2	42.5	4251.6	4.3		
	10	1	0.401	160870	8.7	21.8	2179.6	2.2	2.1	0.0
		2	0.406	160542	8.7	21.5	2149.8	2.2		
		3	0.403	151236	8.4	20.8	2080.7	2.1		

Appendix J :

Mean biomass and % DM of spinach leaves (mean weight of ten plants) fertilised with different concentrations of NH_4NO_3 and grown under ambient CO_2 conditions in a controlled environment chamber.

NH_4NO_3 concentration (mM)	Biomass of spinach leaves (g/plant FW)	Mean biomass (g/plant FW)	SE	Spinach leaves (g DM)	% DM	Mean % DM	SE
1	2.26	2.1	0.3	1.65	27	28.2	0.2
	2			1.42	29		
	1.98			1.31	34		
	1.45			0.97	33		
	2.45			1.69	31		
	2.69			2.18	19		
	3.69			2.84	23		
	1.15			0.76	34		
	0.99			0.7	29		
	2.59			1.99	23		
2	2.78	3.7	0.3	1.81	35	31	0.2
	3.91			2.74	30		
	4.44			3.06	31		
	1.78			1.21	32		
	3.47			2.46	29		
	4.56			3.28	28		
	3.44			2.44	29		
	3.54			2.3	35		
	4.21			3.01	28		
	4.99			3.39	32		
3	2.89	3.8	0.4	2.02	30	32.3	0.2
	2.69			1.94	28		
	4.58			2.93	36		
	4.69			3.19	32		
	4.14			2.77	33		
	1.39			0.92	34		
	5.78			3.53	39		
	4.32			3.07	29		
	4.11			2.88	30		
	2.99			2.03	32		

Appendix K :

Mean total and soluble oxalate levels of spinach leaves fertilised with different concentrations of NH_4NO_3 and grown under ambient CO_2 conditions in a controlled environment chamber.

Extraction	NH_4NO_3 concentration (mM)	Replicates	Sample weight (g)	Peak Area (Uv.s)	Oxalic acid (mg/100mL)	Oxalic acid (mg/g)	Oxalic acid (mg/100 g)	Oxalic acid (g/100 g)	Mean oxalic acid (g/100 g)	SE
Acid (total)	1	1	0.406	620202	29.2	72	7198.8	7.2	7.6	0.2
		2	0.407	662201	31.5	77.4	7742.7	7.7		
		3	0.406	660001	31.4	77.3	7732.3	7.7		
	2	1	0.402	519381	23.7	59.1	5905.4	5.9	5.7	0.1
		2	0.405	504639	22.9	56.6	5663.6	5.7		
		3	0.401	482298	21.7	54.2	5416.9	5.4		
	3	1	0.403	470176	21.1	52.3	5226.3	5.2	5.4	0.2
		2	0.405	522229	23.9	59	5900.0	5.9		
		3	0.404	464121	20.7	51.3	5131.8	5.1		
Water (soluble)	1	1	0.406	566201	23.7	58.3	5829.5	5.8	5.8	0.3
		2	0.400	592106	24.6	61.6	6155.5	6.2		
		3	0.401	502103	21.3	53.1	5313.5	5.3		
	2	1	0.401	504902	21.4	53.4	5339.2	5.3	5.3	0.0
		2	0.403	510128	21.6	53.6	5360.5	5.4		
		3	0.406	512509	21.7	53.4	5342.5	5.3		
	3	1	0.405	379042	16.8	41.4	4142.0	4.1	4.1	0.0
		2	0.401	371467	16.5	41.1	4113.7	4.1		
		3	0.403	369449	16.4	40.7	4074.9	4.1		